STUDIA MEDICA SZEGEDINENSIA

EDIT UNIVERSITAS SCIENTIARUM MEDICINAE SZEGEDINENSIS

REDIGIT AUXILIO CONSILII SCIENTIAS PROMOVENDAS DELECTI
BÉLA KORPÁSSY

TOMUS 1. FASCICULUS 2.

STUDIES ON UROBILINOIDS

BY
I. L. KAHÁN

SZEGED
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INTRODUCTION

Already in the XIX-th century the use of classical chemical methods promoted knowledge concerning chemistry and biochemistry of bile pigments. Much of this still holds good up-to-date. Some of it is also useful in clinical diagnosis.

The modern analytical and preparative chemical methods, e. g. the application of chromatography and electrophoresis, drew attention to the existence of new bile pigment compounds. It was revealed that bilirubin, reacting „directly" with the Hijmans van den Bergh reaction (1928) used since 40 years in clinical diagnosis, is a bilirubin glucuronide. Its chemical structure, biochemical behaviour, physiological role and pathophysiological significance differs significantly from the bilirubin giving an indirect reaction. The questions relating to this problem represent a new trend in bilirubin research work which has proved to be very productive from the physiopathologic and clinical point of view.

On applying the classical methods for the determination of urobilinoids it already became conspicious that is some cases stercobilin, in others rather i-urobilin was excreted and could be found in the urine. It was attempted to use this finding to distinguish jaundice based on impairment of the liver function from haemolytic jaundice, but it became clear that the excretion of these compounds depends on differences in the intestinal flora.

The methodical problems arising in the course of the investigation of these questions directed my attention towards the conjugated urobilinoids the examination of which only became possible by the application of modern methods like chromatography and electrophoresis. In this connection the examination of the electrophoretic behaviour of the urobilinoids furnishes data relating the structure of urobilinoid molecule.

This work was prompted in 1955 by an advise of the late Professor G. HETÉNYI and it is His Memory it should be dedicated to.

Collection of available references was terminated in 1959.

1. HISTORY

JAFFE (1868) observed for the first time in the presence of zinc ions the green fluorescence of the urobilinoids. As he found the substance in bile and urine he termed it „urobilin". In 1871 a compound behaving similarly was found in faeces (van LAIR et al.) and this was called „stercobilin". In the same year still another product obtained by the reduction of bilirubin and behaving in the same way called „hydrobilirubin" was described (MALY 1871). EHRLICH (1901) detected the colour reaction of the chromogens of the urobilinoids with p-dimethylaminobenzaldehyd.

HANS FISCHER et al. (1911) was the first to obtain pure chromogen in a crystalline state by the reduction of bilirubin. When this was exposed to air and light it was converted to urobilinoid which showed a green fluorescence in the presence of zinc ions. This substance gave with Ehrlich's „aldehyd" reagent a coloured product showing a similar absorption spectrum as when urine reacted with p-dimethylaminobenzaldehyd. In the course of the reaction the bilirubin furnished the chromogen with a yield of 50 per cent, hence Fischer called the reduction product first „hemobilirubin" and only later „mesobilirubinogen". FISCHER (1911) also obtained urobilinogen from urine which proved to be identical with the mesobilirubinogen obtained from bilirubin.

WATSON (1932) was the first to obtain crystalline stercobilin from faeces. WATSON (1934), LEMBERG (1934), as well as FISCHER, HALBACH and STERN (1935), furthermore LEMBERG, LOCKWOOD and WYNDHAM (1938) succeeded in proving the difference between this compound and mesobilirubinogen obtained from i-urobilin. The compound forming from the reduction of bilirubin and being transformed after exposure to air into i-urobilin proved to be less stable than the stercobilin isolated from faeces, whilst its absorption maximum differed only to a small extent from the latter. The i-urobilin was inactive, whereas stercobilin proved to be a marked laevorotatory compound.

SCHWARTZ and WATSON (1942) found a third dextrarotatory urobilinoid, d-urobilin. In the course of 1945—50 BAUMGARTEL gave an account of his theory about the formation of stercobilin and urobilin, associating FISCHLER's (1908) hepatogen theory with v. MÜLLER's (1887, 1892) and Mc MASTER's and ELMAN's (1925) enterogen theory (a detailed discussion of this subject can be found in chapter 8). WATSON confirmed his enterogen theory with two experiments (LOWRY et al. 1954; WATSON et al. 1956): he converted N^{15} labelled urobilinogen into stercobilinogen in vivo and converted d-urobilinogen into i-urobilinogen in vitro.

An outstanding progress of urobilinoid chemistry in the following years was the elucidation of the chemical and physical properties of d-urobilin. In 1955 WATSON and co-workers succeeded in isolating d-urobilinogen from faeces. At the clarification of the formula it became obvious that d-urobilinogen is isomer with dihydromesobilirubinogen. In the same year the infra red spectra of all three substances were determined

and x-ray analyses were carried out too (WATSON, et al, 1956). In 1957 GRAY described the structural formula of d-urobilin (GRAY et al. 1957 b., 1958 c.). GRAY succeeded in isolating an other dextrarotatory urobilinoid the formula of which agreed with i-urobilin, as well as an optically inactive modification which had a formula identical to that of d-urobilin (GRAY et al. 1958 b., c.).

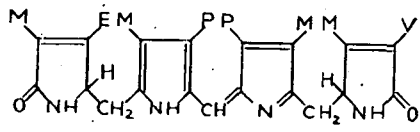
The investigations of Japanese authors dealing with a so far unknown form of urobilinoids in the organism, the urobilinoid-esters, should still be mentioned (NORO, 1951).

2. NOMENCLATURE

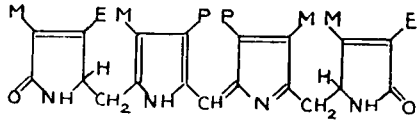
Those bile pigments which are „urobilin-like" compounds are classified as urobilinoids because their physical and chemical properties resemble those of the primarily discovered substance, then called urobilin.

In this group three urobilinlike compounds found in human urine, faeces and bile: d-urobilin, i-urobilin and stercobilin, as well as a few synthetic products are ranged.

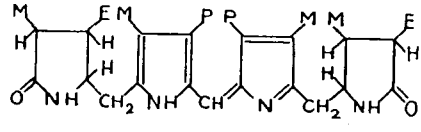
d-urobilin
d-dehydrourobilin



i-urobilin
urobilin IXα
„K" urobilin
mesobilen-b



stercobilin
l-stercobilin
l-urobilin
tetrahydromesobilen-b

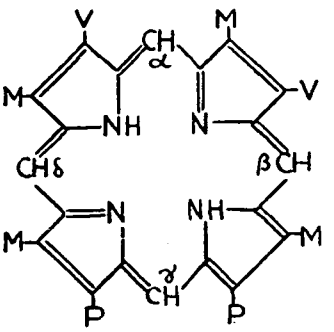


Clue of the symbols hear and in the followings:

M = $-CH_3$ E = $-CH_2-CH_3$ V = $-CH = CH_2$ P = $-CH_2-CH_2-COOH$

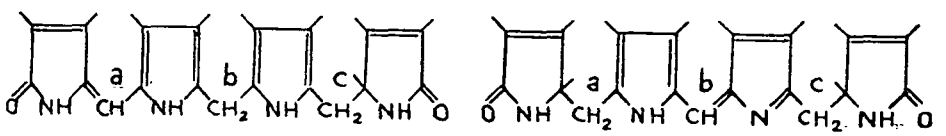
In the literature the terminology of these compounds differs considerably. As the knowledge about the properties of the compounds increased the names varied accordingly. The first denotations were created according to the occurrences of the compounds. As mentioned above (s. chapter 1) i-urobilin as it was first found in urine and bile was named „urobilin" and the similar substance found in the faeces stercobilin. Nowadays we know that the denotation according to the occurrence, and mainly the differentiation on this basis is not justified and not correct: i-urobilin and stercobilin both occur in urine as well as in faeces. The name stercobilin as the name of a different chemical substance is only used since the optical activity of stercobilin is known (FISCHER, HALBACH, STERN, 1935). The name urobilin IXα employed by SIEDEL who synthesized this compound for the first time (SIEDEL, MEIER, 1936) was previously

used for i-urobilin, but it is still employed too. The mark IX means that the arrangement of the side chains in the molecule of the i-urobilin (and in the other native bile pigments) is in agreement with the position of the side chains in the molecule of the protoporphyrin IX and the denotation α indicates that the protophyrin ring split at the α-methin bridge.



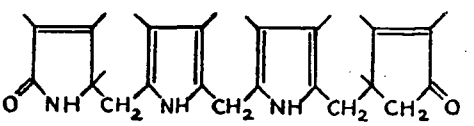
Previously the name K-urobilin indicating a synthetic preparation („künstlich") (FISCHER, MEYER-BETZ, 1911) was used for i-urobilin.

Following the footsteps of SIEDEL (SIEDEL, 1935, SIEDEL and MEYER, 1936) LEMBERG and LEGGE (1949 a.) endeavoured to find a rational term. According to the systematic nomenclature the urobilinoids belong to the group of the bilenes composing the bilene-b group. -ene denotes the double bond in the connecting chain and -b the exact denotation of the position of the double linkage.



bilene-a bilene-b

The rational name already makes use of the closer knowledge of the structure of these compounds: thus the bile pigments with a tetrapyrrolic chain containing two central pyrrol rings conjugated by a methin group belong to the urobilinoid group.

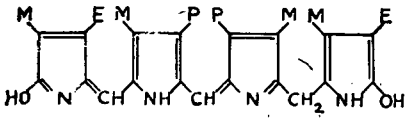


bilan

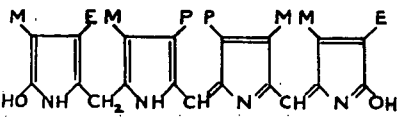
Accordingly the chromogens of the urobilinoids not containing in their conjugating chain a double linkage are bilans.

The oxidation products of the urobilinoids containing two or three double-linkages are the biladiens (mesobiliviolin and mesobilirhodin), and bilatrien (glaucobilin, or by another name mesobiliverdin), respectively.

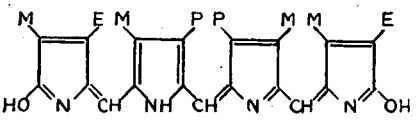
For the designation of the side chains the rational terminology uses the mark applied in the case of the porphyrin ring. The bile pigments contaning, similarly to mesoporphyrin, besides methyl and propionic acid radicals an ethyl side-chain are called „meso" compounds. The rest of the compounds contain like protoporphyrin intsead of an ethyl, a vinyl group. Hence, according to the rational nomenclature i-urobilin is denoted „mesobilen-b" and stercobilin „tetrahydromesobilen-b". The corresponding chromogens, i-urobilinogen and stercobilinogen — which do not contain in the conjugating chain double linkages — are mesobilan and tetrahydromesobilan respectively. (The former, considering that it is also a chromogen of mesobilirubin was also called mesobilirubinogen.)



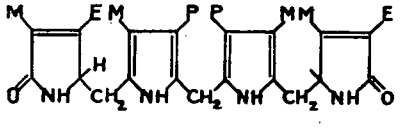
mesobiliviolin
biladien a., b.



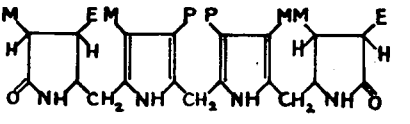
mesobilirhodin
biladien b., c.



glaukobilin, mesobiliverdin
bilatrien



i-urobilinogen; mesobilirubinogen
mesobilan



stercobilinogen
tetrahydromesobilan

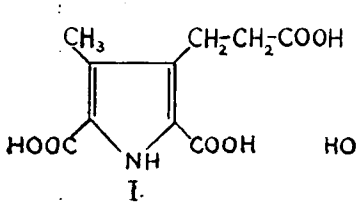
Of course, this nomenclature, claimed by LEMBERG and LEGGE as very much simplified, could not be applied in every days use because it is very complicated. Thus the names urobilin, stercobilin and d-urobilin remained.

Recently GRAY has reported the preparation of two new urobilinoids, about a dextrarotatory form of i-urobilin which he calls „d-urobilin IXα" and a racem „d-urobilin" (GRAY, 1958 b.). To emphasise the difference in the structure still more WATSON and LOWRY (1956) suggests the denotations „i-urobilin", „l-stercobilin" and „d-dehydro-urobilin".

For the sake of simplicity and as the native hitherto isolated urobilinoids contain these three urobilinoids, in this work the terms i-urobilin, stercobilin and d-urobilin will be used.

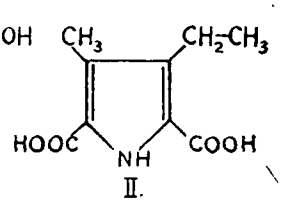
3. THE STRUCTURE OF UROBILINOIDS

Biliverdin develops through the oxidative splitting of protoporhyrin IX. Biliverdin is reduced to bilirubin and then to the chromogens of the urobilinoids which are converted on exposure to air to the corresponding urobilinoid pigments. (This procedure is discussed in detail in chapter 8.) The changes in the structure of the substances taking place in the course of these conversions are shown in Figure 1. It has been proved by means of synthesis that the porphyrin ring actually splits at the α-carbon atom and that the known urobilinoids are formed at its reduction. At synthesis, only the compounds containing a side chain arranged as in porphyrin IX yielded urobilin corresponding to the native i-urobilin (SIEDEL, 1935). GRAY et al. (1958a), confirmed by direct route structure IXα. They oxidised urobilinoids and other native bile pigments in an alkaline medium with potassium permanganate and compared the obtained oxidation products with authentic 3-methyl-4-carboxyethylpyrrol-2,5-dicarbonic acid; 3-methyl-4-ethylpyrrol-2,5-dicarbonic acid and 3-methyl-pyrrol-2,4,5-tricarbonic acid.



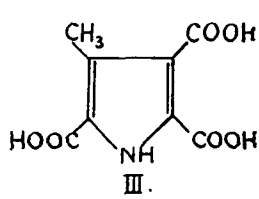
I.

3-methyl-4-carboxy-ethylpyrrol-2,5-dicar-bonic acid



II.

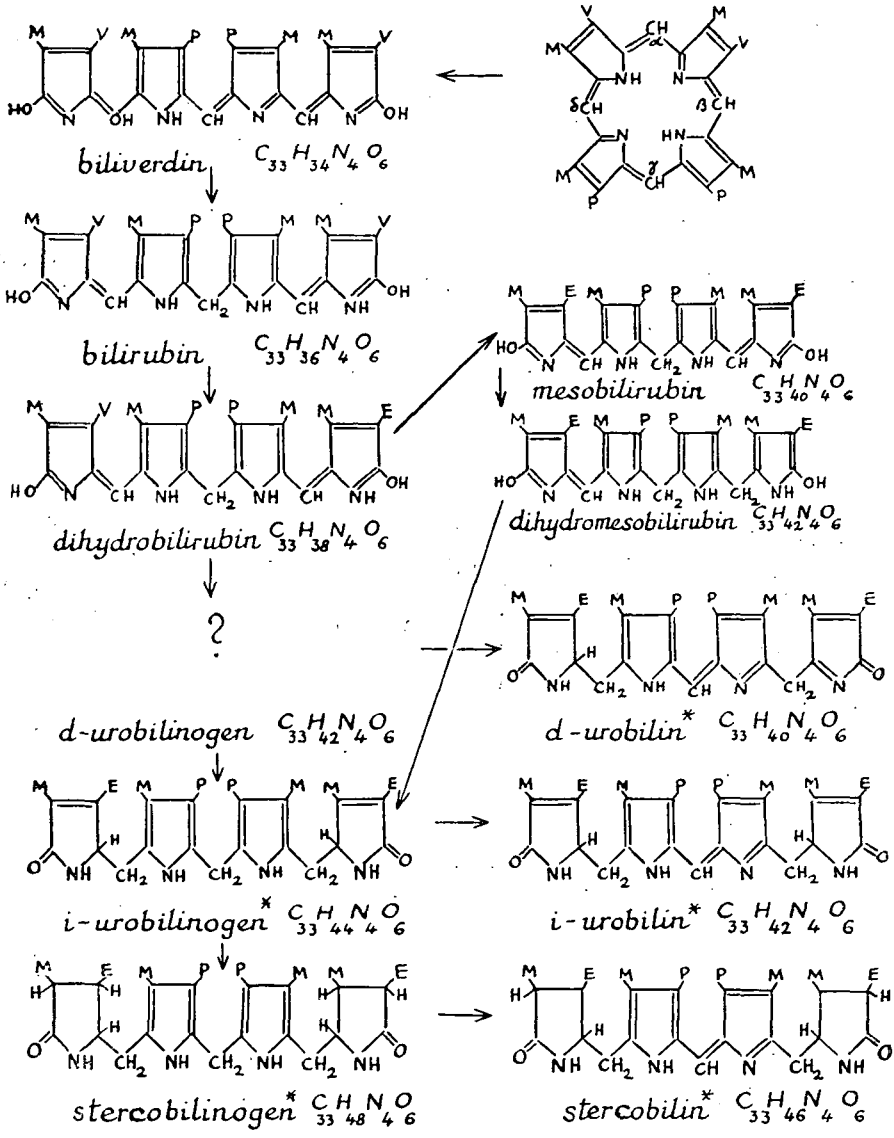
3-methyl-4-ethylpyrrol-2,5-dicarbonic-acid



III.

3-methylpyrrol-2,4,5-tricarbonic acid

In the course of the oxidation always I was formed never II and III. The product of I corresponds to the structure of protoporphyrin IX, whereas those of products II and III to that of some other isomer.

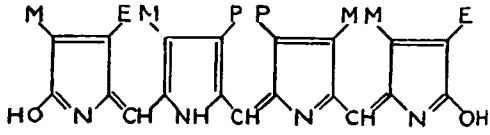


$M = -CH_3$, $E = -CH_2-CH_3$
$V = -CH=CH_2$, $P = -CH_2-CH_2-COOH$,

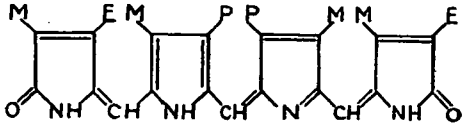
Fig. 1.

Conversion of protoporphyrin IXα to bile pigments.
* Represented as „lactam" formule according to GRAY.

Structure IXα i. e. the tetrapyrrol containing the 1,3,6,7-tetramethyl-2,8-diethyl, -4-5 carboxy-ethyl side chain (the ethyl radical can be substituted by a vinyl radical too) is characteristic.



Concerning the two pyrrol end rings of the urobilinoids the position of the double conjugation is still open to debate. Instead of the earlier lactim form GRAY suggests the lactam one, accordingly the urobilinoids belong to the group of the bilenons:



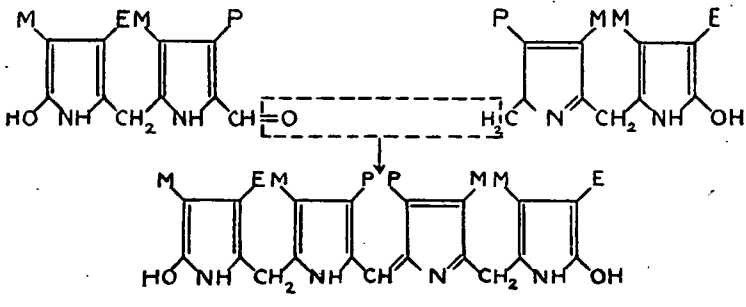
The lactam formula of these compounds has been demonstrated by GRAY by the CrO_3 oxidation of stercobilin (GRAY et al. 1957 a.). GRAY succeeded in isolating a dextrarotatory d-urobilin IXα which hence contains an asymmetric carbon atom. An asymmetric carbon atom can only be present in the urobilin IXα (i-urobilin) lactam formula. (GRAY 1958, b., c.) But for the double linkages situated in the two middle rings the different urobilinoids form through reduction of the double conjugations or by tautomerization.

In the following the structure and physico-chemical properties of the different urobilinoids will be described.

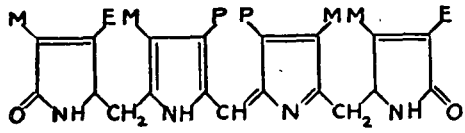
i-Urobilin, Urobilin IXα $C_{33}H_{42}N_4O_6$

The substance crystallises in needles or prisms Mp. 159—164°C. The Mp. of its hydrochloric acid salt is 175—177°C. It is optically inactive. (WATSON and LOWRY 1956).

i-Urobilin is the only urobilinoid the structure of which has been confirmed by synthesis. SIEDEL synthesized it from formylneobilirubinicacid and isoneobilirubinicacid according to the following scheme:

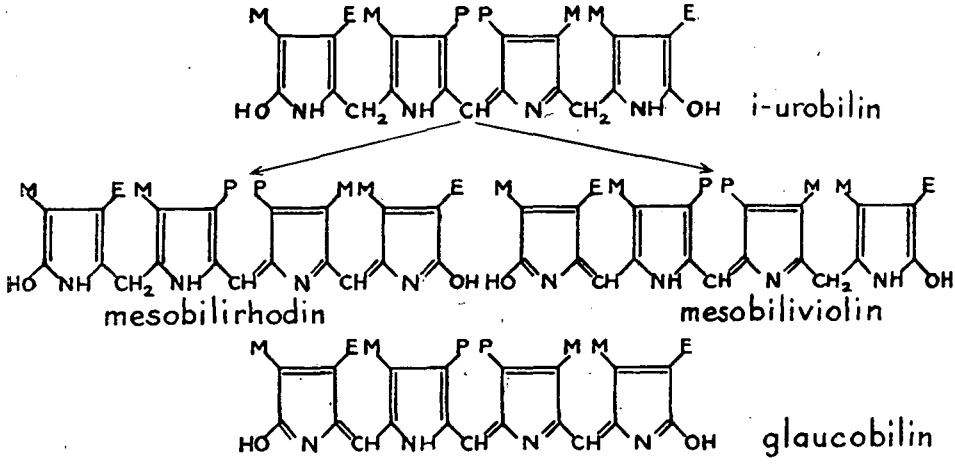


Its structure displayed according to GRAY's so-called lactam formula:



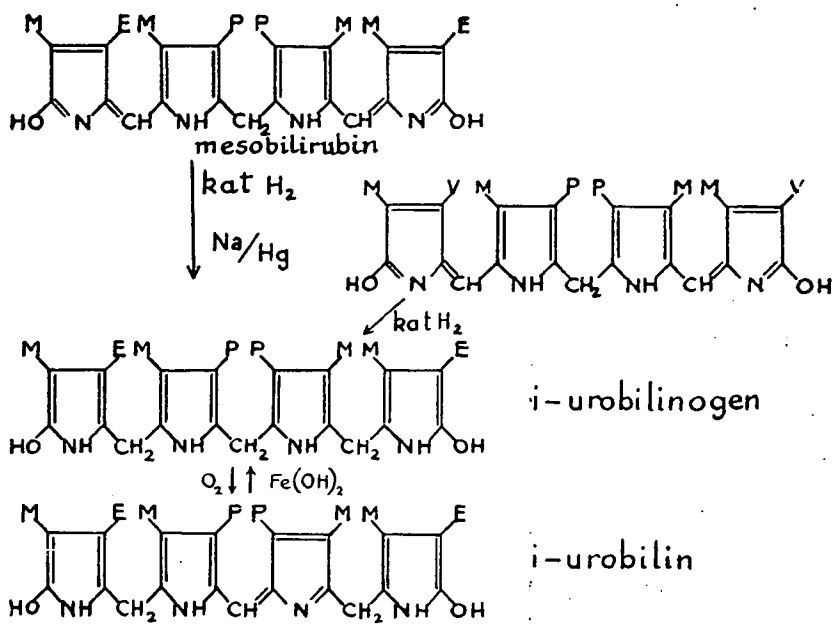
This latter formula which is the only i-urobilin formula containing an asymmetric C-atom is supported — as mentioned above — by the fact that GRAY succeeded in obtaining dextrarotatory urobilin IXα as follows: he reduced d-urobilin and the resulting tetrahydro-d-urobilin (authentically identified with mesobilirubinogen) was converted by mild oxidation to the above mentioned dextrarotatory urobilin IXα. Therefore, the hitherto known i-urobilin can only be a meso- or racem compound.

By oxidation of i-urobilin with $FeCl_3$ a mixture of mesobiliviolin and mesobilirhodin is obtained (LEMBERG, 1934). Further oxidation yielded glaucobilin.

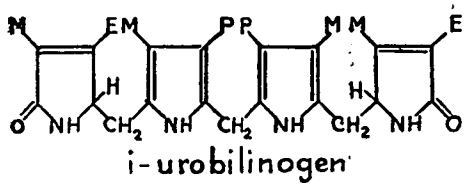


i-Urobilinogen, mesobilirubinogen, $C_{38}H_{44}N_4O_6$

This compound is the chromogen of i-urobilin (urobilin IXα). It is a colourless crystalline substance Mp.: 197—202°C. It can be prepared from mesobilirubin by catalytic hydrogenisation or by reduction with sodium amalgam from bilirubin by catalytic hydrogenisation (FISCHER, 1911, 1915), (FISCHER and MEYER BETZ 1911). If it is exposed to air it is readily converted to i-urobilin, whereas with $Fe(OH)_2$ the i-urobilin can be reconverted to the chromogen.



The lactam formula of i-urobilinogen (Gray, 1958c) is:

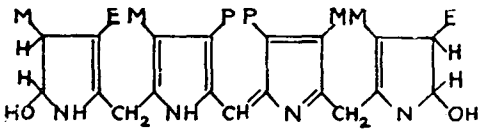


With p-dimethylaminobenzaldehyd (Ehrlich's reagent) i-urobilinogen gives a red condensation product.

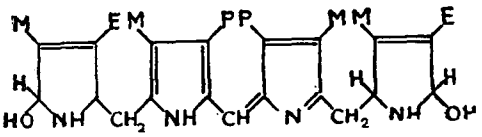
Stercobilin $C_{35}H_{46}N_4O_6$

Mp.: 157—162°C. The Mp. of its mono- and dihydrochloride is 234—236°C (WATSON, and LOWRY, 1956). It is strongly laevorotatory. The optical activity of its hydrochloric acid salt in a solution of chloroform is: $[\alpha]\frac{20}{589} = -3500°$ (FISCHER, HALBACH, 1936; FISCHER et al, 1935) or more recently $[\alpha]\frac{20}{589} = 4000°$ (WATSON, 1952). From its elementary analysis it becomes obvious that it contains 4 more H-atoms than i-urobilin (FISCHER, HALBACH, 1936). As at the oxidative degradation of i-urobilin methylethylmaleinimide was obtained, whereas in the case of stercobilin this breakdown product could not be isolated the position of the additional 4 H and thus the formula of stercobilin changes according to FISCHER and HALBACH (1936)

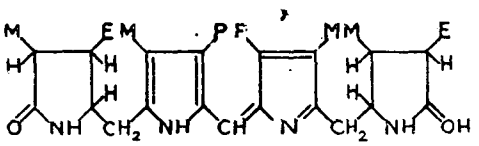
in the following manner:



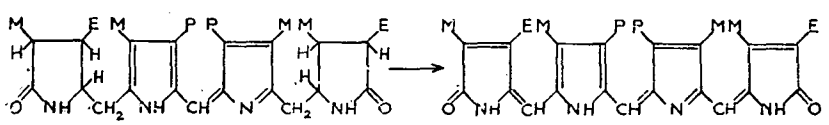
SIEDEL and GRAMS (1940) suggest that the position of the double bonds in the two pyrrol end rings of stercobilin is different from that mentioned above.



In 1955 BIRCH suggests that the formulae mentioned above are not in agreement with the great stability of stercobilin. In 1957 GRAY has described the CrO_3 oxidation of stercobilin. In the course of the degradation he obtained acetic acid, succinic acid, haematinimid and methylethylsuccinicimide. The formation of the latter oxidation product can only be explained by a number of tautomeric structures depending upon the environment, or by the following so-called lactam formula:

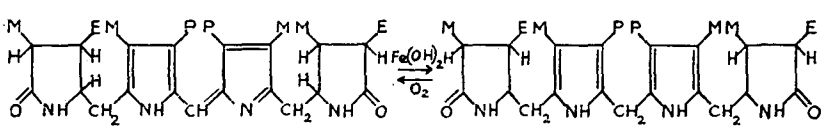


In contrast to i-urobilin stercobilin cannot be dehydrogenated with $FeCl_3$, but it forms a complex with it. This property which i-urobilin fails to show is used at the analysis of the urobilinoids (chapter 5): stercobilin can only be converted into glaucobilin by the action of vigorous oxidation and treatment with hot concentrated H_2SO_4:



stercobilin glaucobilin

On reduction with $Fe(OH)_2$ it gives stercobilinogen which does not crystallise.

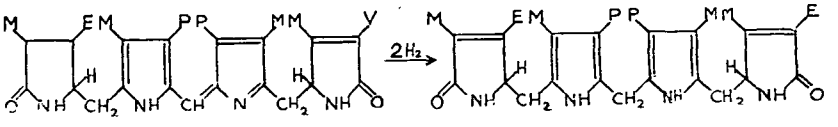


Stercobilinogen $C_{33}H_{48}N_4O_6$

If exposed to air stercobilinogen is reconverted into stercobilin. Like i-urobilinogen stercobilinogen gives with p-dimethylaminobenzaldehyd a red condensation product.

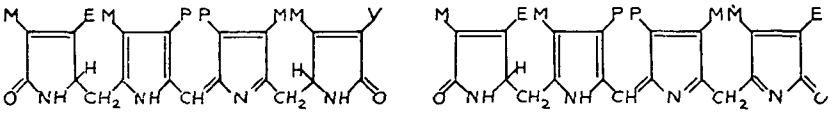
d-Urobilin $C_{33}H_{40}N_4O_6$

Mp.: 162—165°C. Mp. of its hydrochloride: 172—174°C (WATSON and LOWRY 1956). It is strongly dextrarotatory $[\alpha]\frac{20}{589} = +5000°$ (SCHWARTZ et al, 1942) (WATSON, 1956). From its elementary analysis it becomes obvious (LOWRY, et al, 1956) that it contains two less H-s than i-urobilin. Its structure was elucidated by oxidative degradation, hydrogenisation and decisively by isomerisation with alkali (GRAY, NICHOLSON 1957 b., 1958 c.). By oxidation with CrO_3 ethylmethyl-maleinimid was obtained suggesting the presence of a 3-ethy-4-methyl-pyrrol ring. It the course of catalytic hydrogenisation each molecule consumed 2 mol. H_2 and the tetrahydro-d-urobilin thus formed could be identified with mesobilirubinogen.

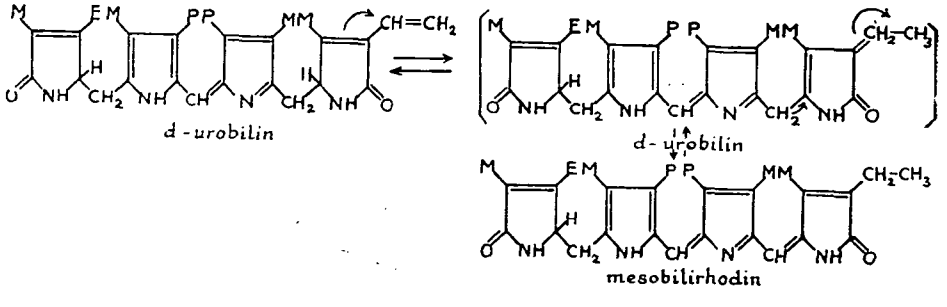


d-urobilin mesobilirubinogen

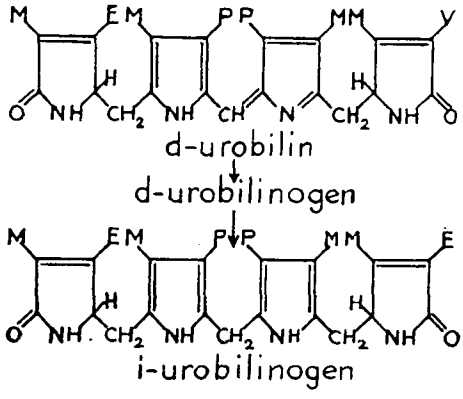
On the basis of the absorption maximum of d-urobilin (WATSON and LOWRY, 1956) and the identity of the formed chromogen with i-urobilinogen GRAY et al, (1958 c.) suggests among several structures in the first place the following two ones:



Similarly to i-urobilin d-urobilin is converted by the action of treatment with $FeCl_3$ to mesobiliviolin and mesobilirhodin. In the case of d-urobilin this conversion also takes place in a reducing medium which can only be conceived to be due to tautomerization. The tautomerization occurs presumably according to the following scheme:



d-urobilin is readily converted to glaucobilin too and at the analysis this property is also made use to differenciate it from the other two urobilinoids. By mild reduction with sodium amalgam d-urobilin can be converted to d-urobilinogen, whereas if the reduction is prolonged i-urobilinogen (mesobilirubinogen) forms.



d-Urobilinogen $C_{33}H_{42}N_4O_6$

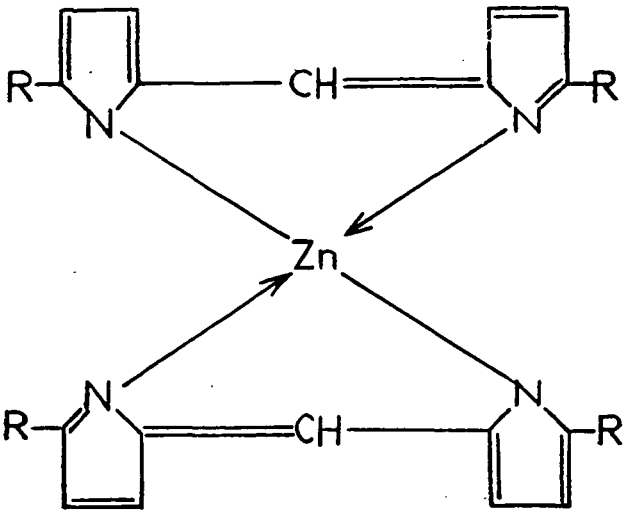
White needle crystals. The Mp. of d-urobilinogen crystallised from chloroform is 110—115°C. d-urobilinogen is mildly dextrarotatory: $[\alpha] = \frac{20}{50} + 74°$ (LOWRY et al, 1956).

In the following the common physico-chemical properties of urobilinoids will be described.

As mentioned above the physico-chemical properties of the urobilinoids are from many standpoints similar. They have particularly three characteristic properties: 1. they are yellow 2. their solutions show a green fluorescence in the presence of zinc ions and 3. their chromogens give with p-dimethylaminobenzaldehyd (Ehrlich reagent) a red colour.

1. Their yellow colour is in accordance with the fact that their absorption maximum corresponds to that of the other dipyrrylmethenes: 250 $m\mu$ and 450—500 $m\mu$ (PRUCKNER, DOBENECK, 1942). The absorption maximum ot their hydrochloric acid salt and chloroform complex shifts towards the long wave lengths. The absorption maximum of i-urobilin and d-urobilin agrees completely (494,5 $m\mu$), whereas the absorption maximum of stercobilin differs slightly (492,5 $m\mu$).

2. The green fluorescence shown by urobilinoids in the presence of zinc is a characteristic feature of the dipyrrylmethenes. 2—2 urobilinoid molecules bind one Zn atom. In this conjugation the secondary N of the central pyrrol ring of dipyrrylmethene participates with a covalent and 2—2 tertiary N dative linkages, thus a tetrahedral structure develops (PORTER, 1938):



If an acid group is introduced into the dipyrrylmethene molecule it inhibits the formation of a Zn complex and thus fluorescence does not either develop (FISCHER, et al, 1939).

3. With p-dimethylaminobenzaldehyd reagent the chromogens of urobilinoids give a red condensation product (EHRLICH, 1901). According to WATSON the absorption maximum of this condensation product is at 560—565 mμ (WATSON, 1936). Optimally the reaction takes place at pH 0,15 — 0,87 (YAMAOKA et al 1956).

4. ELECTROPHORETIC BEHAVIOUR OF THE UROBILINOIDS

Although as early as 1932 (BENNHOLD) the question arose whether urobilinoids conjugated to serum protiens migrate in the electric field, systematic investigations dealing with this subject have not been carried out.

In 1958 it was observed that crystalline urobilinoids not conjugated to the proteins do migrate in the electric field (KAHÁN I. L., 1958). This paper only deals with the electrophoretic behaviour of i-urobilin and stercobilin. The investigation of the three urobilinoid pigments — of those mentioned above and of d-urobilin — as well as the examination of their zinc complexes, their chromogens, and that of their oxidation products is interesting both from the point of view of structure research and preparative reasons. Hence we investigated the followings:

I. The electrophoretic behaviour of the urobilinoid pigments (d-urobilin, i-urobilin and stercobilin).

II. The electrophoretic behaviour of the urobilinoid-Zn complexes.

III. The electrophoretic behaviour of i-urobilinogen (mesobilirubinogen).

IV. The electrophoretic behaviour of the oxidation products (mesobiliviolin, glaucobilin).

V. Finally, results obtained by means of the above investigations were adapted for preparative purposes.

Methods

1. The urobilinoid pigments were dissolved in chloroform. 0,5 ml (5—50 μg/ml) urobilinoid was applied to a paper strip (the same procedure was used for the chromogen).

2. For the investigation of the Zn complexes of the urobilinoids 0,01 ml/ml of an alcoholic saturated Zn acetate solution was added to the solution prepared according to 1. 0,05 ml of this solution was like in the case of solution 1. applied to the paper strip.

3. Buffer solutions:

At pH $\geqq$ 5,0 Michaelis'barbital-sodium acetate–HCl buffer (μ = 0,1 of ionic strength).

At pH $\leqq$ 5,0 sodium acetate — acetic acid buffer (μ = 0,1 of ionic strength).

4. The electrophoresis was carried out on 4 × 40 cm horizontally placed paper strips at 120 V and 400 V(3V/cm or 10 V/cm.)

For the electrophoresis Schleicher-Schüll No. 2043 b paper was used. The three urobilinoids were separated two by two on each paper strip. All combination possiblities were examined.

For continous electrophoresis 350 V (10 V/cm) Whatman No. 4 paper and 10 hours separation time were used.

5. Development: On the electrophoretic paper strips dried at room temperature the urobilinoids can be well detected owing to their yellow colour. After spraying with saturated alcoholic Zn acetate solution (SCHLESINGER 1903) even traces of urobilinoids showing a green fluorescence could be observed in ultraviolet light. The Zn complexes also show a green fluorescence without being developed. The oxidation products can be recognised by their colour. The i-urobilinogen (mesobilirubinogen) was developed with p-dimethylaminobenzaldehyd reagent (EHRLICH 1901).

Results and discussion

I. *The electrophoretic behaviour of the urobilinoid pigments (d-urobilin, i-urobilin and stercobilin).*

The results of the examinations carried out at 120 V are shown on Fig 2. on which the electrophoretic migration values of the three bile pigments are illustrated as a function of the pH.

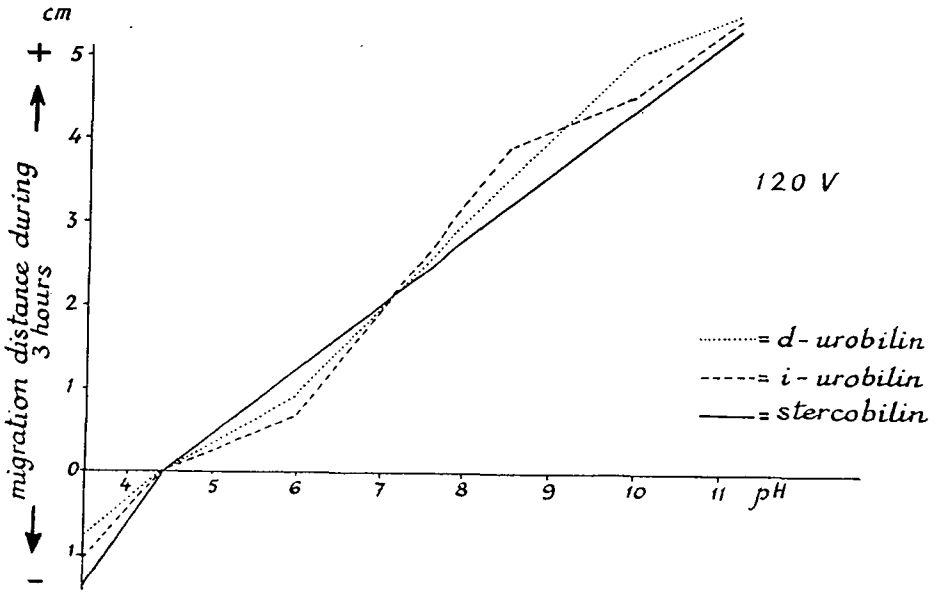


Fig. 2.

Electrophoretic behaviour of d-urobilin, i-urobilin and stercobilin. The electrophoretic migration is shown as a function of the pH. 120 V (3V/cm), Schleicher Schüll No. 2043 b paper, Michaelis barbital-sodium acetate hydrochloric acid buffer at pH>5,0 and M/5 sodiumacetate-acetic, acid buffer at pH<5,0 both with an ionic strength of $\mu = 0{,}1$ were employed.

It may be seen that in some respects the three bile pigments behave similarly in the electric field. At pH 4,4 the compounds do not move from the starting point. This pH value may be considered to be their isoelectric point. Below it all three

urobilinoids behave like cations, above it like anions. As it is known that i-urobilin and stercobilin are present even at their isoelectronic point in an ionised state (LEMBERG, LEGGE, 1949 b.) it must be assumed that the equilibrium of the basic and acid groups is at pH 4,4. Under this pH the direction of the migration is determined by the basic groups above it by the acid ones.

Whereas, within the limits of error of measuring, the isoelectric points of all three urobilinoids were the same and above and below this pH the three compounds behaved identically regarding the direction of their migration, the extent of their migration differed.

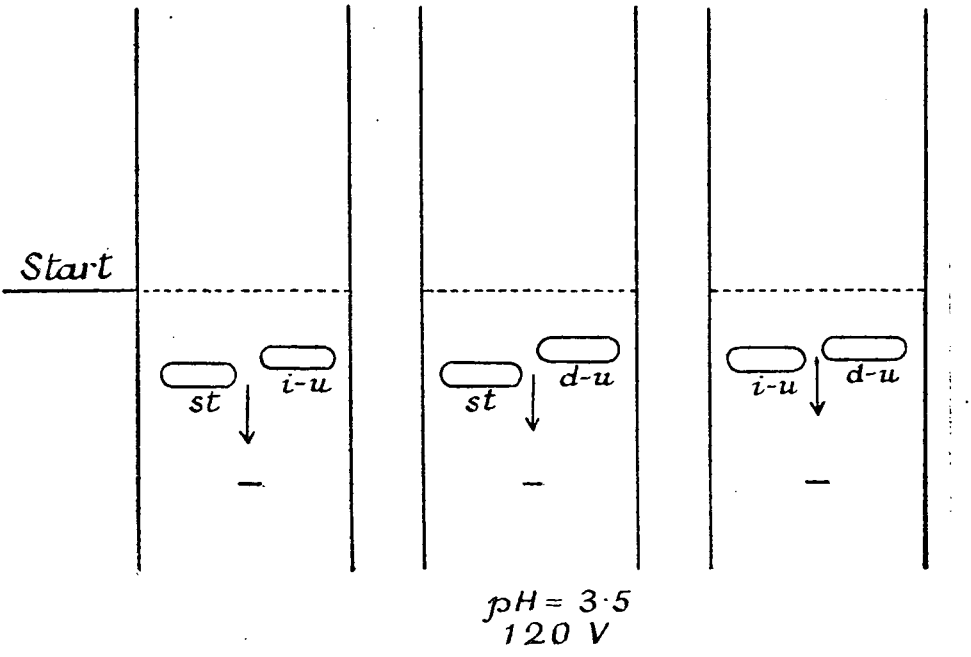


Fig. 3.

Schematic diagram of electrophoretic migration of urobilinoids at pH 3,5. On the paper strips 2 urobilinoids were applied each. 120 V (3V/cm), Schleicher Schüll No 2043 b paper sodium-acetate hydrochloric acid buffer and an ionic strength of $\mu = 0{,}1$ were employed.

As can be seen in the Fig. 2 and Fig. 3 below the isoelectric point the mobility of stercobilin is the greatest and that of d-urobilin the slightest, the mobility of i-urobilin ranges between that of stercobilin and d-urobilin. In the range of pH 4,4— pH 6,9 the sequence of mobility of the compounds is as follows: stercobilin, followed by d-urobilin and finally by i-urobilin. In the range of pH 6,9 — pH 7,1 the compounds migrate at the same rate. In the range of pH 7,0 — pH 9,2 i-urobilin migrates the fastest it is followed by d-urobilin and than comes stercobilin. At pH 9,2 the migration rate of d-urobilin and i-urobilin is the same whereas that of stercobilin is the slowest.

Finally in a pH range above pH 9,2 the rate of the d-urobilin migration is the fastest it is followed by i-urobilin whilst that of stercobilin is the slowest (Fig. 4).

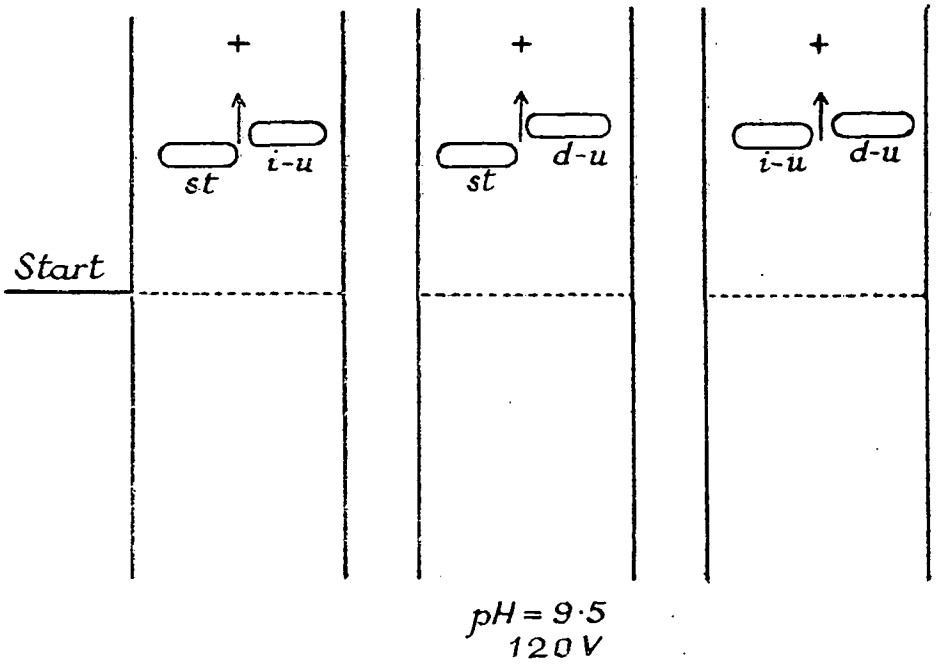


Fig. 4.

Schematic diagram of electrophoretic migration of urobilinoids at pH 9,5. On the paper strips 2 urobilinoids were applied each. 120 V (3V/cm), Schleicher Schüll No 2043 b paper Michaelis barbital sodiumacetate hydrochloric acid buffer and an ionic strength of $\mu = 0{,}1$ were employed.

For the explanation of the electrophoretic behaviour the following points of views may play a role:

In chapter 3 the structures of the urobilinoids were described: accordingly all three compounds contain propionic acid radicals, heteroaromatic basic nitrogens. Of the three compounds stercobilin is the most saturated. i-Urobilin contains in the end rings 2—2 double bonds whereas d-urobilin contains still an additional double linkage. In view of the fact that according to our investigations the isoelectric point is in the range of the acidic pH, in all three compounds the acid character is stronger than the basic one. As regards the sequence of the migration below the isoelectric point the extent of the movement is proportional with the basic character. It is the greatest in the case of stercobilin and the slightest in that of d-urobilin. Indeed the NH-groups situated in the end rings of the i-urobilin molecules belonging to the conjugated double bonded system are less basic than the corresponding NH groups in the end rings of stercobilin behaving as ordinary amids. Whereas in the d-urobilin molecule the conjugation of the double bonds increases, even as compared to the i-urobilin. The results of the experiments carried out below the isoelectric point are in good agreement with these considerations.

In the case of pH values above the isoelectric point the electrophoretic pattern is more complex. Presumably here the contrary of the conditions below the isoelectric

point ought to be observed. This only occurs at extremely high pH where the migration rate of d-urobilin is the greatest, then follows i-urobilin and finally stercobilin. On Fig. 2 it is visible that at 120 V, below pH 9,2, the urobilinoids change the sequence of their migration rate several times and that in a certain pH range it is identical for all three urobilinoids. This phenomenon drew the attention to the fact that besides dissociation another factor changing with the pH — presumably adsorption — plays a role at the development of the migration rate. This certainly also depends upon the quality of the paper used for the electrophoresis, as well as on the structure of the urobilinoid molecule, thus upon the number and position of their double bonds too. To diminish the influence of the adsorption the electrophoresis was carried out at higher voltage. It can be seen in Fig. 5 that at 400 V the result of the experiment concerning the sequence of the migration differs from the previous one:

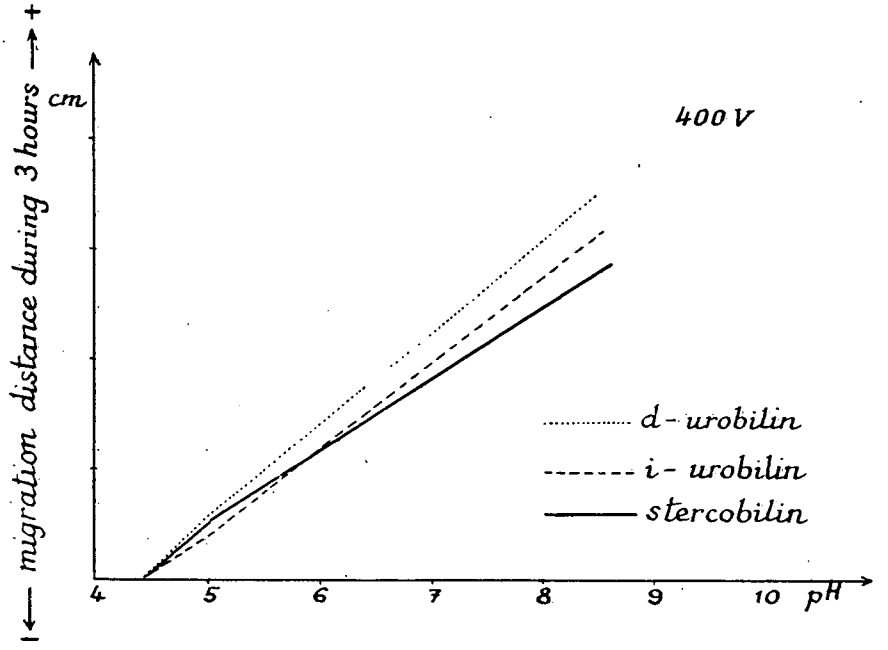


Fig. 5.
Electrophoretic behaviour of d-urobilin, i-urobilin and stercobilin. The electrophoretic migration is shown as a function of the pH. 400 V (10 V/cm), Schleicher Schüll No 2043b paper Michaelis barbital sodiumacetate hydrochloric acid buffer at pH>5,0 and M/5 sodiumacetate acetic acid buffer at pH<5,0 both with an ionic strength of $\mu = 0{,}1$ were employed.

Already above pH 6,0 the electrophoretic pattern expected on the basis of the structure was obtained: the migration of the least basic molecule d-urobilin was the fastest, it was followed by i-urobilin and finally by stercobilin. Above the isoelectric point quite until pH 5,0 d-urobilin migrated together with stercobilin. Hence it can really be assumed that in the case of paper electrophoresis the adsorption influences the migration of the urobilinoids to a great extent. We succeeded in considerably diminishing the effect of the adsorption by increasing the voltage used

for the electrophoresis, this phenomenon can, however, still be observed at 400 V: it decreases the mobility of d-urobilin — the least basic molecule — only to a small extent, merely in the vicinity of the isoelectric point, above pH 5,0 the influence of dissociation is stronger than that of adsorption, and among the three urobilinoids the migration rate of d-urobilin is the greatest. The mobility of i-urobilin till pH 6,0 even at 400 V is still strongly influenced by the adsorption. Above this pH value an electrophoretic pattern corresponding to the structure of the molecule is obtained.

Thus, if the adsorption is taken into account most of the phenomena may be interpreted. (KAHÁN I. L. et al, 1959 c.). The influence of the adsorption is stronger at both Volt numbers around the isoelectric point where the difference in the chemical character is less dominating that at higher pH where the dissociation is decisive. Of course, below the isoelectric point using both voltages the conditions are the same because the smaller the number of the double bonds the greater the basicity, the smaller the adsorption and the greater the readiness of migration. Hence dissociation and adsorption influence in the same way the rate of migration of the molecules. Thus as concerns the structure of the compounds the electrophoretic data support the assumption that of the three urobilinoids d-urobilin is the most acidic molecule and stercobilin the least acidic one.

Our observations regarding the behaviour of the three urobilinoids in the electric field — in the first place in the pH ranges where the dissociation is complete, i. e. below pH 4,0 and in the range around pH 9,0 — are not only in agreement with the theoretical considerations mentioned above, but also with the spectroscopic measurements aiming to determine of the dissociation constant, as carried out earlier by GRAY (1953 a) with i-urobilin and stercobilin. According to these pK 7,0 for i-urobilin and pK 8,0 for stercobilin indicating that i-urobilin is a compound of more acidic character than stercobilin.

As regards d-urobilin such spectroscopical investigations have not been performed. Our experimental results are in accordance with the proposed structural possibilities, but a decision cannot be reached. Concerning the suggested structural formula of d-urobilin this molecule may be considered to have a less basic character than i-urobilin, but a more basic one than mesobiliviolin. This is in agreement with the fact that at the electrophoretic run mesobiliviolin — which is a more acidic molecule than d-urobilin migrates faster than d-urobilin does (see IV.).

II. *Electrophoretic behaviour of the zinc complex of the urobilinoids*

The electrophoretic behaviour of the zinc complex of urobilinoids was examined at pH 9,0 and at pH 6,0. (At lower pH the complex is unstable.) It was found that the zinc complexes do not migrate in the electric field, however, after a certain time the urobilinoid pigments are released by the action of the electric field from their complex conjugations and thus they migrate as zinc-free urobilinoids. In the electric field the stability of the zinc complexes is, however, not similar and changes according to the applied pH and applied urobilinoid. The difference between the stability of the stercobilin-zinc complex and that of the i-urobilin-zinc complex was well visible. If a voltage of 120 V is applied at pH 8,6 the decomposition of the-i-urobilin-zinc complex can already be noted after about half an hour and after two hours it is complete, whereas the greater part of the stercobilin zinc complex remains even after an electrophoretic run lasting for seven hours without decomposing at the position of the application (Fig. 6.).

With a buffer pH 6,0 the result of the electrophoresis is different. Curiously enough at this pH the stability of the i-urobilin zinc complex is greater as compared to that of the stercobilin zinc complex. The stability of the d-urobilin zinc complex could be found in both pH ranges between that of the other two urobilinoids. In ultraviolet

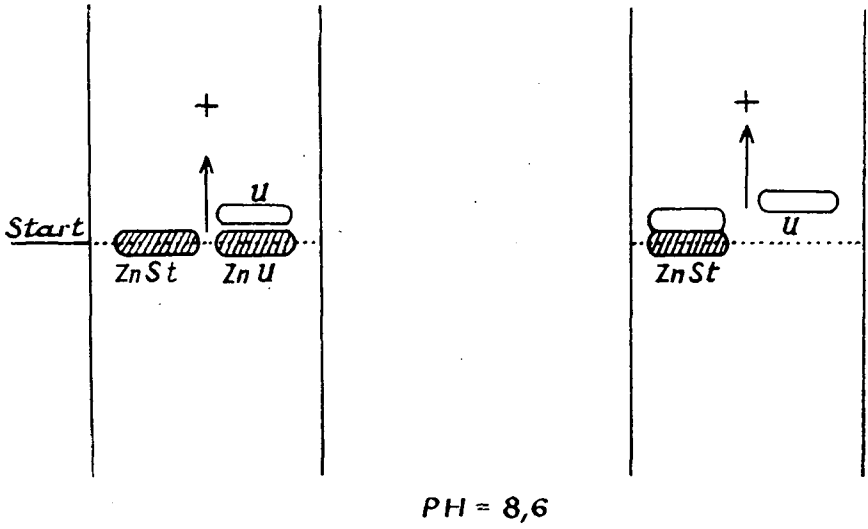


Fig. 6.
Schematic diagram of the electrophoretic behaviour of the zinc complexes of the urobilinoids at pH 8,6. 120 V (3V/cm), Schleicher Schüll No 2043 b paper, Michaelis barbital-sodiumacetate hydrochloric acid buffer and an ionic strength of $\mu = 0{,}1$ were employed. Left: after ½ hours, right: after 2 hours run.

light the Zn complexes showed a green fluorescence without having been previously sprayed with Schlesinger reagent. For the reproducibility of the experimental results the concentration of the urobilinoid solutions had to be identical and their concentration with respect to zinc must be the same too. If the zinc concentration was different the results varied and if too little zinc was used this also resulted in the appearance of the „multi-spot" phenomenon as mentioned in regard to other complexes earlier (PUCAR, 1957).

III. *Electrophoretic behaviour of i-urobilinogen (mesobilirubinogen)*

Among the chromogens of the urobilinoids crystalline i-urobilinogen (mesobilirubinogen) was chosen for systematic investigations.*

On running i-urobilinogen in the usual Michaelis barbital-sodiumacetate-HCl buffer (pH 8,6) it could be observed that after a relatively short period of separation (about two hours) 2 yellow spots proceeding towards the anode became visible on the paper. On spraying them with Schlesinger reagent they showed a green fluorescence. The spot running fastest showed a weaker green fluorescence (that was to enhance with oxidation of iodine), whereas that one nearer the start showed a stronger one.

* Other urobilinoid chromogens isolated from urine showed a similar behaviour.

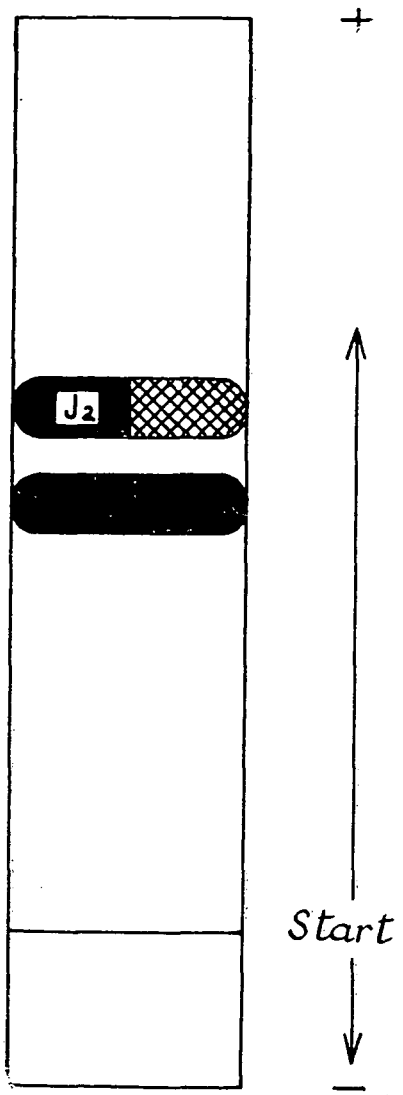


Fig. 7.
Schematic diagram. Characterisation of the electrophoretic migration of urobilinoids and their chromogens at pH 8,6. 120 V (3V/cm), Schleicher Schüll No 2043 b paper, Michaelis barbital sodium-acetate hydrochloric acid buffer and an ionic strength of = 0,1 were used. Schlesinger positive: ■ p-Dimethyl-aminobenzaldehyd reagent positive: ⁞⁞⁞

The position of the latter agrees with that of the simultaneously applied i-urobilin reference. On carrying out the development according to PARTRIDGE (1948) with p-dimethylaminobenzaldehyd reagent the spot nearer the anode showed a red colour characterizing the chromogens of urobilinoids (Fig. 7).

The fact that two urobilinoid spots can be demonstrated on the paper and that the position of the one is in agreement with that of the simultaneously applied i-urobilin reference, whilst the other gives a colour reaction also with p-dimethylamino-benzaldehyd reagent suggests that partly during the migration on the paper and partly in the course of the drying following the electrophoresis i-urobilinogen is oxidized to urobilinoid pigment.

On carrying out the electrophoresis at lower pH the electrophoretic migration rate decreases steadily in the case of the i-urobilinogen like in that of the urobilinoid pigments until it caesas at pH 4,2. If the pH is still decreased the electrophoretic picture remains unchanged: at pH 4,2 i-urobilinogen does not leave the starting point by the action of the electric field. The dissociation of i-urobilinogen owing to it being a weaker base than the urobilinoid pigments (LEMBERG, LEGGE, 1949 c.) is completely repressed at pH 4,2 or below this value.

IV. *The electrophoretic behaviour of the oxidation products (mesobiliviolin, glaucobilin).*

The oxidation products of the urobilinoids were not available to us in a crystalline state, but considering that they play a role at the electrophoresis and analysis of the urobilinoids it seemed worth while to investigate their electrophoretic behaviour.

On separating the urobilinoids at 400 V and pH 7,2 for 3 hours their oxidation and tautomerization products form separate bands, i. e. in front of i-urobilin and d-urobilin the violet band characterising mesobiliviolin ap-

pears and in front of stercobilin a pink band becomes visible. If the electrophoresis was carried out at a lower pH (pH 5,0), in front of i-urobilin and d-urobilin the green band characterising glaucobilin and in front of stercobilin a pink band appeared. These products were extremely labile and elready decomposed on the paper in the course of the drying, hence further examinations could not be carried out.

On evaporating an etheric mixture of mesobiliviolin-mesobilirhodin prepared by the oxidation of i-urobilin with $FeCl_3$ in the way described in chapter 5 and submitting it to paper electrophoresis it could be observed that the substance remains for a longer period (1—2 hours) on the site it was applied, and also if 250—280 V was used, the characteristic pinkish red (zinc complex of mesobiliviolin) and yellow (Zn complex of mesobilirhodin) fluorescence could only be demonstrated (with Schlesinger's reagent) after an electrophoresis of 4—5 hours in a position between the start and the anode. Hence the conclusion may be drawn that the metal complexes of mesobiliviolin and mesobilirhodin respectively (presumably the Fe-complex formed from the $FeCl_3$ used at the oxidation) behave similarly to the Zn complexes of the urobilinoids. They do not migrate in the electric field, but they decompose by the action of the electric field and similarly to the urobilinoids, the mesobiliviolin and mesobilirhodin released from the complex migrate as anions.

V. *Electrophoresis adapted for preparative purposes.*

For the chemical preparation of the urobilinoids (i-urobilin from bilirubin) or their isolation from faeces or urine the purity of the products obtained must be controlled. It was found that besides chromotography, electrophoresis is also suitable for this purpose. The urobilinoids separate very well from other bile pigments. On using barbital buffer at the customary pH 8,6 bilirubin remains at the site of application and the urobilinoids migrate towards the anode, whereas their oxidation products precede them.

Homogenous and pure urobolinoid can easily be obtained. Smaller amounts are obtainable by *paperelectrophoresis* in the following manner: The substance to be purified is applied to 5—6 paper-electrophoretic strips. The electrophoretic paper strips are dried at room temperature, cut out on the suitable places (on the place of the simultaneously run reference substances) and cut into small pieces and then eluated. For the eluation barbital or borate buffer similar to that used for the run, or hydrochloric acid-alcohol, or hydrochloric acid-chloroform may be employed. Paper remnants were discarded by means of a glass filter gauge 12 G 4.

For the isolation of somewhat larger amounts of urobilinoid a *continuous electrophoretic apparatus* was used. On applying 350 V (10 V/cm) the urobilinoids can be separated from the bilirubin as well as from the oxidation products in a manner resembling that described above. The urobilinoids dropping down are diluted with the applied buffer and may be extracted from this solution after acidification with chloroform.

5. ANALYTICAL METHODS

In this chapter the analysis of urinary urobilinoids mainly their quantitative analysis, will be dealt with, the determinations carried out with faecal and biliary urobilinoids agree in principle with those performed with the urinary ones thus they will not be considered in detail. The determinations accomplished with serum will be discussed in a separate chapter.

Most urobilinoid estimations determine total urobilinoids. These estimations are based 1. on the measurement of the red colour of the chromogens which is formed with p-dimethylaminobenzaldehyd 2. on that of the yellow colour of the urobilinoid pigments or 3. on the measurement of the green fluorescence of the Zn complexes of the urobilinoid pigments.

The latter method is the most sensitive and thus for the measuring of small amounts of urobilinoid, e. g. serum urobilinoids, it is the only adequate procedure as will be described later. For measuring the urobilinoids in the urine, however, the two first less sensitive methods are also adequate. Considering that the determination in the form of chromogens involves the danger that owing to the oxidation a part of the urobilinoids will not be determined and, in addition, the colour reaction yielded by p-dimethylaminobenzaldehyd is not specific, the determination of the total urobilinoids as well as the procedure described below which was elaborated for the separate estimation of urinary i-urobilin and stercobilin were based on the measurement (KAHÁN, I. L. 1957) of the yellow colour of the urobilinoid pigments. The same principle was applied by WATSON (1959).

Numerous qualitative methods have been elaborated for the isolated investigation of urinary i-urobilin and stercobilin partly based on the difference of the spectrum of the copper complexes of these two substances (FISCHER, HALBACH, 1936) and partly on the oxidation of the i-urobilin by the action of hydrogenperoxide to pentdyopent, whilst the stercobilin remains unchanged (BINGOLD, 1941). The optical activity of stercobilin has also been used for the isolated investigation of urobilinoids (FISCHER et al., 1939; FISCHER et al., 1935; FISCHER, HALBACH, 1936; HALBACH, 1938).

For the isolation and estimation of the urobilinoids and their oxidation products chromatography on talcum columns was attempted (SIEDEL, 1935; EISENREICH, 1948), and recently paper chromatography was also tried (KEHL, 1952, 1954; GOHR et al., 1956), but as the R_f values of these two very similar substances differ only slightly this kind of partition was not even appropriate from the qualitative point of view. For the latter purposes most of the procedures so far applied (BAUMGARTEL, 1950 a; MAGYAR et al., 1955) made use of the fact that i-urobilin oxidises by the action of $FeCl_3$ to mesobiliviolin, whereas at the same time a yellow iron complex forms from stercobilin (cf. chapter 3) (FISCHER, et al., 1924; HOESCH, 1926; LEMBERG, 1934; SIEDEL, MOELLER, 1940).

In the method described below the urinary urobilinoids were separately determined by the so-called „mesobiliviolinreaction" mentioned above by adapting the method of LEGGE (1948) for faecal urobilinoids to urine (KAHÁN, I. L., 1957).

Description of the procedure:

A quantity of 50—200 ml urine, depending upon the daily amount of urine excretion and the urobilinoid content to be expected, was kept standing in the air for a day at pH 4,5 so that the urobilinogen and stercobilinogen should oxidise to i-urobilin and stercobilin respectively. It was shaken in a separatory funnel with chloroform until colourless. The extracts were dehydrated with sodium sulphate and evaporated to dryness in vacuum. The residue is taken up in so much ethanol (5—125 ml) that the extinction of the solution may be measured with a Pulfrich photometer using a S_{50} filter. The measuring gives the sum of the i-urobilin and stercobilin. Then an aliquot part (5 ml) of the ethanolic solution is oxidised with 0,5 ml 6 M HCl being lemon yellow from ferric chlorid and heated

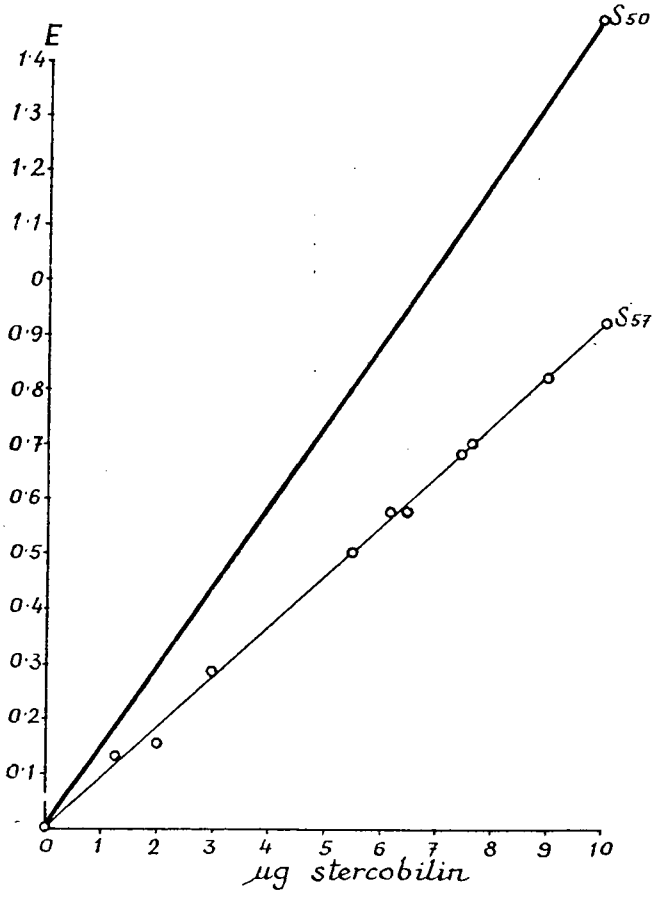


Fig. 8.

Calibration curves for quantitative estimation of i-urobilin and stercobilin taken up before and after oxidation with $FeCl_3$. Extinction values of extracts taken up in ethanol measured at 550 mµ before and at 570 mµ after oxidation. (S_{50} and S_{57} filter of Pulfrich Stufenfotometer respectively). d = 1 cm.

on the water bath to 15 minutes. The so formed products, i. e. the stercobilin iron complex and the oxidation products of i-urobilin mesobiliviolin and mesobilirhodin are shaken into peroxide-free ether. The iron complex of stercobilin is washed out with water, whilst mesobiliviolin and mesobilirhodin are shaken into 2,8 N HCl from the ether and the extinction of the violet solution is measured with a S_{57} filter. By this measurement the quantity of the i-urobilin is determined, whereas by the subtraction of the values corresponding to the extinctions S_{50} and S_{57} the quantity of stercobilin is estimated by means of calibration curves (Fig. 8.).

Considering that a part of the urobilin is already easily oxidised by the oxygen of the air, the calibration curve used for the measuring of the yellow colour of the ethanolic solution containing the urobilinoids was prepared with stercobilin. The stercobilin was crystallised from faeces according to the method of FISCHER and LIBOWITZKY (1939) and its homogenity was controlled by electrophoresis. The i-urobilin used for the calibration curve was prepared from bilirubin according to Fischer's reduction method (1911). Mesobiliviolin extinctions of the ten urine samples from which stercobilin-iron could not be washed out by our method coincided with the extinction values of the mesobiliviolin curve obtained from crystalline i-urobilin. Hence the yellow substance determined by means of the S_{50} curve gives the quantity of the i-urobilin (Fig. 8.), when stercobilin is not present.

Discussion and some further details of the method

a) Stercobilinogen and i-urobilinogen are excreted with the urine. On standing in air they are spontaneously oxidised to stercobilin and i-urobilin, respectively. This oxidation can be promoted with $CuSO_4$—HCl or J_2, but in every oxidation method the danger is involved that in the course of the oxidation i-urobilin is further oxidised to mesobiliviolin and mesobilirhodin, or by the action of iodine still further, i. e. to oxourobilin. According to our investigations standing for one day is optimal to obtain i-urobilin and stercobilin from their chromogens. On standing two days the loss is greater, at least 5,5 per cent of the urobilin is oxidised to mesobiliviolin. Extraction is continued until the urine does not react with Ehrlich's reagent.

b) The extraction procedure: At pH 4,5 was found to be optimal. As urobilinoids are coloured substances it is well visible that after shaking, the best staining of the chloroform layer with the compounds investigated can be achieved at this pH.

c) Adding of different amounts of standard i-urobilin and stercobilin to normal urine and by determination of the total urobilinoids, it was attempted to elucidate the extent of the urobilinoid loss in the course of shaking urine with chloroform and evaporating it to dryness.

Table 1.

i-Urobilin and stercobilin loss during the extraction procedure

No. of experiment	Urobilin + stercobilin measured in normal urine µg/100 ml	Added		Total i-urobilin + stercobilin		Deviation in percent
		i-urobilin µg/100 ml	stercobilin µg/100 ml	calculated µg/100 ml	found µg/100 ml	
1.	23,8	11,3	—	35,1	33,8	— 11
2.	10,8	—	5,0	15,0	14,0	— 20

in the case of i-urobilin and stercobilin concentrations exhibited in the Table 1. the calculated and found total urobilinoid values are fairly approximate.

d) The oxidation with ferric chloride. This is the most sensitive step of the procedure, thus it is most important to keep strictly to the required conditions (concentration of the hydrochloric acid containing $FeCl_3$, time of oxidation, temperature). Under such conditions only mesobiliviolin and mesobilirhodin (i. e. biladiens) form. At still higher temperature or greater concentration of $FeCl_3$—HCl still further dehydrogenation takes place and glaucobilin (bilatrien) forms. In the course of our experiments this only occurred once when reflux condenser was not adjusted and the temperature as well as the concentration of the $FeCl_3$—HCl rose higher than needed. To elucidate the optimal conditions the concentration and the time of application (30 minutes) of the $FeCl_3$—HCl was deliberately enhanced. Glaucobilin formed which on shaking with chloroform gave it a green colour. The fact that under the defined appropriate conditions glaucobilin does not form even in traces and only mesobiliviolin and mesobilirhodin result was evidenced by chromatography on a talcum column according to SIEDEL (1935). Only a brownish-red ring corresponding to mesobilirhodin and a violet one corresponding to mesobiliviolin were obtained. As the absorbtion maxima of the two substances are close to each other in a hydrochloric acid medium it is possible to measure the sum of the two substances with a S_{57} filter.

To control the quantitative conditions of the oxidation step 60 μg/ml i-urobilin was added to i-urobilin-free normal urine. After carrying out the extractions and the mesobiliviolin reaction 50 μg/ml i-urobilin was found which proved the usefulness of the above two steps of the procedure. The same is demonstrated by the fact that if different amounts of the same urine sample were determined the deviation did not surpass in the case of the total urobilinoids 5 per cent and in that of i-urobilin 15 per cent.

e) Impurities. It must be taken into account that owing to the extinction of the urochroms present, a larger amount of urobilinoid can be measured than present. Therefore in the case of strongly coloured urines it is advisable to shake the i-urobilin and stercobilin from the chloroform extracts with 25 per cent HCl, and then to reshake the 4x diluted hydrochloric acid solution into purified chloroform. The impurities giving an extinction with a S_{50} filter do not any more gain access into this solution. Great disturbances are caused if larger amounts of bilirubin passes through and get into the chloroform extract of the urine. This is also shown by the fact that with filter S_{47} the extinction is higher (the absorption maximum of bilirubin is at 450 mμ) than with filter S_{50}, whereas in the case of i-urobilin and stercobilin it is 8 per cent lower with filter S_{47}.

It should be attempted to bind the bilirubin impurity already before the extraction with freshly prepared calcium phosphate precipitate, according to the method of JENDRASSIK and GRÓF (1938) whereby the filtrate only shows a weak colour with the diazo reagent. The urobilinoid loss occurring in the course of the adsorption is shown on Table 2.

Table 2.

i-Urobilin and stercobilin loss in the course of preparing bilirubin free urine

No. of experiment	Urinary stercobilin + i-urobilin mg%	i-urobilin mg%	Added bilirubin mg%	After removal of bilirubin			
				stercobilin + i-urobilin		i-urobilin	
				mg%	deviation%	mg%	deviation%
1.	0,62	0,12	0,85	0,58	— 6	0,14	+17
2.	0,62	0,12	0,34	0,50	—20	0,12	0
3.	0,62	0,12	0,17	0,54	—13	0,12	0
4.	0,62	0,12	0	0,61	— 1	0,11	— 9

It can be seen that the loss never exceeds 20 per cent.

As described in the last chapter the daily normal stercobilin + i-urobilin output ranged between 0,3—1,5 mg. The i-urobilin amounts at the utmost to 30 per cent of the total urobilinoids, whereas under pathological conditions sometimes values about 20 mg/die were measured.

Our procedure elaborated for the determination of urinary urobilinoids seems to give reproducible results; WATSON (1959) obtained similar results with a theoretically analogous method.

6. CONJUGATED URINARY UROBILINOIDS

Urinary urobilinoid determinations (KAHÁN I. L., 1957) carried out with the chloroform extraction procedure (LEGGE, 1949) may owing to two reasons give lower values than the true ones. On shaking with chloroform, the deep staining of the protein emulsion at the interface became visible pointing to the fact that a part of the urobilinoids was bound to it. Besides this, urobilinoid fractions are determined only if they are present in a chloroform soluble form (NORO, 1951). This was also proved by the observation that if some of the urine samples were shaken with chloroform any length of time the extracted urine always contained urobilinogen (KAHÁN, I. L. et al, 1959, a., b.).

Methods

I. *Extraction methods:*

1. Shaking with chloroform-amylalcohol

For the separation of the urobilinoids soluble in chloroform and insoluble in it as well as for that of those bound to the protein emulsion the shaking of the urine (immediately after the excretion) for one hour with a 1/5 volume of a chloroform amylalcohol mixture (9 : 1) proved to be the most suitable (SEVAG, 1937). After removing the top phase of the three phases separating after centrifugation for 15 minutest at 3000 r. p. m. it was extracted 20 times with 1/3 volume of ethylacetate, then the extracts were evaporated together and this yielded the urobilinoid fraction insoluble in chloroform. On removing with a spatula the strongly stained middle protein emulsion phase it was either dissolved at pH 8,6 $\mu = 0{,}1$ in barbital buffer, or the emulsion was mixed with ethylacetate added dropwise on a watch glass. The emulsion collapsed, became colourless and its ethylacetate extract could be poured off after centrifugation, the supernatant was used for the examination. — The lower phase containing the chloroform soluble fraction was once more centrifuged and pipetted from beneath the residue of the emulsion and evaporated in vacuum.

2. Shaking with ethylacetate

Urine was shaken with ethylacetate in portions of 1/3 volumen until the concentrated last extract was negative when tested for urobilinogen. The purified centrifuged combined extracts were evaporated in vacuum at 40°C. After removing in bulk

the ethylacetate extract the residue was sharply centrifuged; thus it separated into three phases as follows: a very narrow ethylacetate supernatant, a protein emulsion and a lower aqueous phase. The ethylacetate phase was sucked first with a glass capillary and then with a filter paper and the centrifuged tube was cut in the region of the lower limit of the emulsion layer. The emulsion separated in this manner was dissolved in barbital buffer (pH 8,6). At this procedure the extracted urine always becomes urobilinogen negative.

3. $(NH_4)_2SO_4$ butanol extraction

According to the method elaborated for the extraction of unconjugated bilirubin and esterified one, (HEIKEL, SIPILA, TENHUNEN, 1957), urine was saturated at room temperature with $(NH_4)_2SO_4$, 2 per cent butanol was added and it was vigorously shaken. After standing for 10 minutes it was centrifuged, where 4 phases developed:

a) A top butanol phase with a deep colour which is sucked with a glass capillary and evaporated in vacuum at 40°C and then centrifuged once more.

b) A fatty lustrous top protein membrane; this is removed with a spatula, and is either dissolved in barbital buffer (pH 8,6), or mixed with butanol or ethylacetate added drop by drop on a watch glass and washed till it becomes colourless.

c) Colourless aqueous urobilinogen negative phase.

d) Stained protein prepcipitate at the bottom of the test tube. As this only differed in its colloid properties, but not in its composition from the top membrane it was usually not examined.

II. *Paper chromatography*

1. The fractionation of the chloroform soluble chloroform insoluble and protein bound urobilinoids.

For this purpose a) ascending chromatography was carried out on Whatman No 3 paper for one hour; solvent: water saturated chloroform : butanol : pyridin (2 : 2 : 1) and

b) descending chromatography on Schleicher-Schüll No 2043 b paper for 3 hours; solvent: acetylacetate : chloroform : aceton (1 : 1 : 1) saturated with water (the lower phase was used).

2. For the identification of the sugar derivatives descending chromatography on Whatman No. 1 paper was carried out for 14 hours in a solvent of butanol : pyridin : water (3 : 2 : 1,5) as suggested for sugar chromatography (CHARGRAFF, 1948).

III. *Paper electrophoresis**

The paper electrophoretic examinations were carried out with an apparatus resembling that described by MAYOU-FLINN, but horizontally stretched Schleicher-Schüll No 2043 b paper strips 250 V (7 V/cm) were used.

1. As the study of the electrophoretic behaviour of the „free" urobilinoids was the least disturbed by other factors (mainly adsorption) at pH = 9,0 $\mu = 0{,}1$ barbital

* A separate chapter is devoted to development — and staining procedures.

buffer and the above voltage number (Kahán I. L. et al., 1959 c.) the results obtained with electrophoresis for 5 hours in the above buffer are reported.

2. For the separation of the free urobilinoids and those bound to different sugar derivatives paper electrophoresis (0,25 M pH 8,6 borate buffer) for 5 hours was used. Namely, in this medium the sugars and sugar derivatives attain a characteristic mobility towards the anode through the formation of a borate complex, where as fractions containing uronic acids proceed at the front (CONSDEN, STANIER, 1952). As controls, the same substances were simultaneously submitted at pH 8,6 $\mu = 0{,}1$ barbital buffer to paper electrophoresis. In the latter the similar behaviour of a fraction which separated itself from the free urobilinoid in the course of borate electrophoresis, to that of the free urobilinoid, points to the presence of a sugar derivative, whereas the partition occurring in the barbital buffer indicates the presence of some non-sugar component. — The movement was compared to that of crystalline stercobilin run simultaneously.

3. Paper elctrophoresis was carried out for 5 hours at pH 8,6 in $\mu = 0{,}1$ barbital buffer to examine the urobilinoid fractions conjugated to proteins and mucoproteins, respectively. If they are run over a longer period part of these compounds decompose.

IV. *The development and staining of the paper chromatograms and electrophoretic strips*

1. The development of the urobilinoids

a) The site of the urobilinoid pigments can already be recognised by their yellowish red colour, but a more sensitive method is the spraying of the paper with Schlesinger reagent (saturated alcoholic zinc acetate), thus the urobilinoid pigments show a green fluorescence in ultraviolet light.

b) For the development of the chromogens the papers are sprayed with p-dimethyl-aminobenzaldehyd reagent (0,1 g p-dimethyl-benzaldehyd dissolved in 3 ml etanol + 3 ml cc HCl + 18 ml butanol) and put for 5 minutes into a 90°C thermostat.

2. The development of sugars and sugar derivatives

a) The development was carried out with the p-dimethyl-aminobenzaldehyd reagent described above (PARTRIDGE, 1948), first treated with alkaline acetylacetone reagent for the development of the hexoamines and then without it for that of the acetylhexosamines (violet colour).

b) The uronic acids were developed with naphtoresorcinol reagent (blue colour) (BRYSON and MITCHELL, 1951).

c) The reducing sugars were developed with anilinhydrogenphtalate reagent (brown, fluorescent spots) (PARTRIDGE, 1949).

3. The staining of proteins, mucoproteins and mucopolysaccharides

a) For the indication of proteins staining by acidic fuchsin (NOVOTNY, 1952) was used.

b) The mucoproteins were developed by means of PAS staining by the method of BJÖRNESJÖ (1955) as modified by LAURELL and SKOG (1956).

c) For the indication of the mucopolysaccharides the iron colloid method (KAHÁN, Á., 1959) based on the selective adsorption of ferriammoniumglicerate and development with Prussian blue (RINEHART, ABUL-HAJ, 1951) were used.

V. *The eluation of the urobilinoid fractions obtained by means of paper electrophoresis and examination of the eluated substances*

1. Eluation

A narrow longitudinal strip of each of several paper electrophoretic strips run simultaneously was developed. Zones corresponding to the site of the urobilinoid fractions were cut out of the undeveloped strip sections, hacked up and let stand for 15 minutes at 80°C with an amount of 0,25 M pH = 8,6 borate buffer uniformly wetting the paper pieces. Then the paper pieces were placed into a 12 G 4 glass filter from which the solution was pressed out with a glass rod, sucked up with vacuum and washed with buffer added drop by drop.

2. Urobilinogen and uronic acid detection from eluate

Urobilinogen demonstration from borate eluate of borate electrophoresis fractions was carried out by addition of cold Ehrlich's aldehyd reagent and the demonstration of uronic acid was performed with carbazol reaction (DISCHE, 1947).

VI. *Examination of the effect of β-glucuronidase*

1. Incubation of urine with β-glucuronidase

a) *Preparation of the urine samples*

For the incubation with β-glucuronidase the strongly urobilinogen positive urine of patients suffering from haemolytic icterus was used. Immediately after excretion they were distributed into test tubes and put for one minute into a hot water bath and then suddenly cooled in an ice water bath. The urines were usually halved, one half was used for the experiment with β-glucuronidase and the other for the control one. Both portions were adjusted to pH = 5,0 with N/10 pH = 5,0 sodium acetate acetic acid buffer solutions.

b) *Enzyme preparations*

To each ml of urine 1000 units of *liver* β-glucuronidase were applied. The enzyme was suspended in a small amount of saline and then 5 ml of a N/10 sodium acetate solution was added. To activate the enzyme 0,03 g desoxyribonucleinic acid previously dissolved in 0,2 ml of 0,5 N NaOH was added. The substance prepared in this manner was added to the urine treated in the way decribed above.

In other experiments 1000 glucuronidase units (prepared from the gastric juice of *helix pomatia*) were applied for each ml urine in the way described above.

c) *Incubation.* The incubation was carried out at 37°C in a nitrogen atmosphere.

d) *Extraction.* The urines treated with enzyme as well as the untreated ones were submitted to ammonium sulphate butanol extraction in order to perform qualitative examinations.

2. Preparation of urinary mucoprotein and incubation with urobilinoid and β-glucuronidase

a) *Preparation of urinary mucoprotein*

Preparation of the urinary mucoprotein was started by means of the procedure elaborated according to Tamm and HORSFALL (1952). (The salting out was performed at 4°C, pH = 6,5 with 0,58 M NaCl. Then the residue which had been washed with NaCl was dialysed, and the supernatant evaporated at 40°C.) Howewer the substance obtained in this manner was *not* dissolved according to Tamm and Horsfall in 0,025 M pH = 6,8 phosphate buffer on centrifuging the undissolved part, but the whole substance which had been suspended in the above phosphate buffer was used.

b) *Incubation with β-glucuronidase and stercobilin*

To a mucoprotein suspension of 0,5 ml, 0,0036 g liver β-glucuronidase (Fluka) dissolved in 0,2 ml 0,025 M (pH 6,8) phosphate buffer was added and kept for an hour at 37°C, then crystalline stercobilin dissolved in saline was added and it was stored for an hour at 4°C.

Results

I. *Three chromatographic fractions of urinary urobilinoids*

1. The chromatographic characterisation of the three fractions separated by extraction

a) The barbital buffer (pH 8,6) solution of the middle protein emulsion which was obtained by shaking the fresh urine with $CHCl_3$-amylalcohol followed by centrifugation remained motionless at the site of application in both chromatographic solvent systems (water saturated $CHCl_3$: butanol : pyridin and etylacetate : chloroform : acetone). This start spot has also urobilinoid pigment content showing a yellow colour and a green fluorescence in ultraviolet light if it is sprayed with Schlesinger reagent. Green fluorescence can only be detected at the start. This start spot is also stained by protein stain (acid fuchsin) mucoprotein developing (PAS) and mucopolysaccharide indicating (iron colloid) procedures.

The urobilinoid pigment cannot be extracted with $CHCl_3$ from the fresh protein emulsion obtained by centrifugation, but after extraction with $CHCl_3$ and treatment with ethylacetate it dissolves. On chromatographying its supernatant, however, it was revealed that it also behaves in quite an identical manner as the original precipitate.

The barbital buffer solution of the protein emulsion formed when fresh urine is shaken with ethylacetate, behaves in the same manner.

b) The lower phase of fresh urine obtained by shaking with $CHCl_3$-amylalcohol and centrifuging behaves in both chromatographic solvent systems like crystalline stercobilin. It migrates at the front in the aqueous chloroform : butanol : pyridin sol-

vent and moves in the ethylacetate : chloroform : aceton solvent between the start and the front (see Table 3., Fig. 9). On spraying with Schlesinger reagent a green fluorescence can only be demonstrated on these sites but on staining with acid fuchsin, PAS, iron colloid, or with anylin hydrogenphtalate the development is negative.

Table 3.

Rf values of the chromatographic fractions of urinary urobilinoids

The denomination of the urobilinoid fraction	Ascending chromatography water saturated $CHCl_3$: butanol : pyridin (2 : 2 : 1) solvent	Descending chromatography ethylacetate : aceton : $CHCl_3$ (1 : 1 : 1) solvent
Urobilinoids bound to protein emulsion forming at shaking with ethylacetate and $CHCl_3$-amylalcohol respectively	0	0
Urobilinoids bound to sugar derivatives	0,64	1,0
Free urobilinoids	1,0	0,22

c) In the case of freshly treated urines on shaking with amylalcohol: $CHCl_3$ the top water phase remained urobilinogen positive with Ehrlich reagent. This $CHCl_3$ not extractable urobilinogen, can be extracted with ethylacetate. Concentrating and applying this ethylacetate extract to chromatography it moves differently to crystalline stercobilin: in aqueous $CHCl_3$: butanol : pyridin solvent it can be detected between the start and the front and in a ethylacetate : $CHCl_3$: aceton solvent at the front. (Table 3., Fig. 9.) On development with Schlesinger reagent the chromatogram shows a green fluorescence only on these sites. This oblong spot cannot be developed by acid fuchsin or PAS staining, with iron colloid staining it becomes slightly visible and with development with anilin hydrogenphtalate used for indicating reducing sugars it becomes brown. Thus the three urobilinoid fractions obtained by shaking with $CHCl_3$-amylalcohol also differ in their chromatographic behaviour. The urobilinoid of the emulsion forming in the course of shaking remains at the site of application and it is bound to proteins and mucoproteins, respectively. The urobilinoid of the lower chloroform phase migrates like the pigment of the „free" urobilinoid and according to the negative results of the applied developments too, it is not bound to either proteins or sugar derivatives. The migration of the top aqueous urobilinoid phase differs from that of the „free" urobilinoid, but according to the development with aniline hydrogenphtalate it is bound to sugars or sugar derivatives.

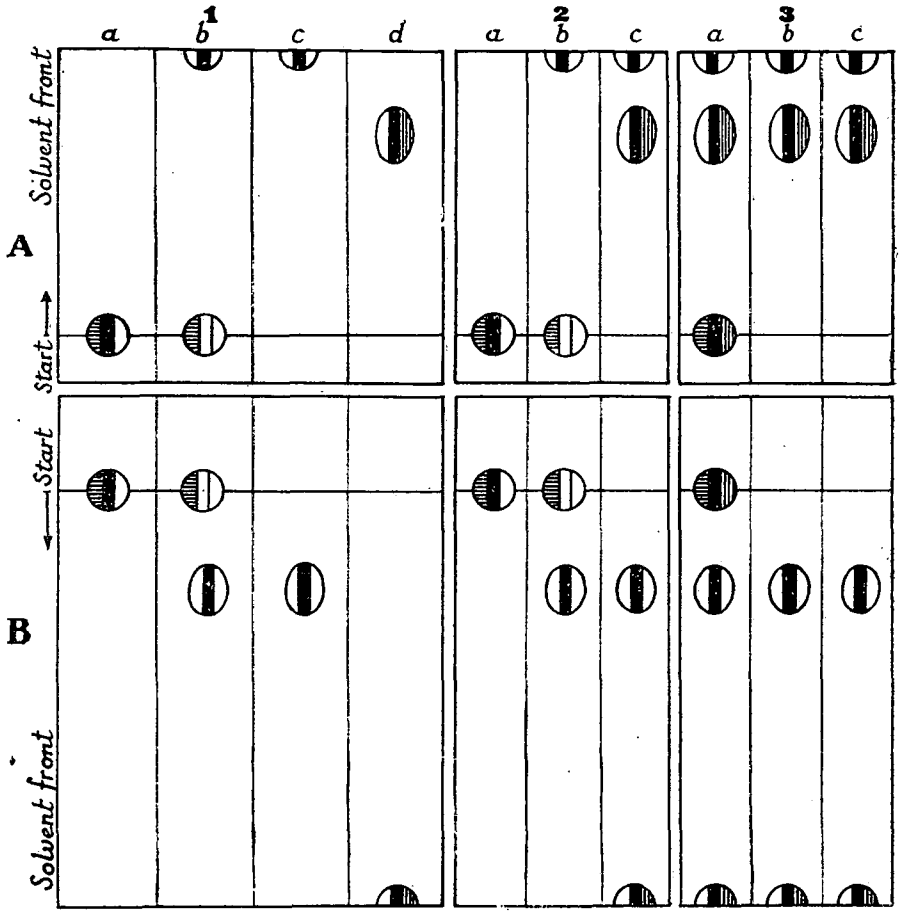


Fig. 9.

The site of the three chromatographic fractions of urinary urobilinoids in the phases obtained with the different extraction procedures. Top row of chromatograms: (A): Ascending chromatography. Solvent = water saturated chloroform : butanol : pyridin (2 : 2 : 1). Lower row (B): Descending chromatography. Solvent = ethylacetate : chloroform : acetone (1 : 1 : 1). Vertical row of chromatograms: 1. fresh protein emulsion formed by shaking with $CHCl_3$-amylalcohol (a); protein emulsion stored for 24 hours (b) lower chloroform phase (c) ethylacetate extract of aqueous top phases (d). 2. Fresh protein emulsion formed at shaking with etylacetate (a); protein emulsions stored for 24 hours (b); ethylacetic top phase (c). 3. Fresh protein precipitate formed at $(NH_4)_2SO_4$-butanol extraction (a); protein incubated with trypsin (b); top butanol phase (c). Fluorescence in ultraviolet light by spraying with Schlesinger's reagent: ▬ Stained with protein and mucoprotein (PAS) staining: ≡ Development with anilinhydrogenphthalate: ||||

2. Chromatographic examination of urine extracts containing several urobilinoid fractions

a) If fresh urine is immediately shaken with ethylacetate and shaking with $CHCl_3$ (or $CHCl_3$-amylalcohol) is omitted, or extraction with $(NH_4)_2SO_4$-butanol is carried out and chromatography is performed in both chromatographic solvent systems described above the repeatedly centrifuged ethylacetate, or butanol extracts proved to contain the mixture of two substances, the one of which moves identically with crystalline stercobilin and the other moving differently being an urobilinoid linked to a sugar derivative. If the latter was heated with anilinhydrogenphtalate it became brown.

b) When the top membrane forming at preparation with $(NH_4)_2SO_4$-butanol is freshly dissolved (pH 8,6) in barbital buffer and chromatographied in the two solvent systems three fluorescent spots appeared on the chromatogram after spraying with zinc acetate. One at the site of application, the second identically with the site of that of the crystalline stercobilin and the third at the site of the urobilinoid moving in a different manner to the crystalline stercobilin. If the latter spot is heated with aniline hydrogenphtalate it shows a brown colour and can also be slightly stained with iron colloid procedure, whereas the spot corresponding to the crystalline stercobilin does not stain. The middle of the start spot may be made visible with acid fuchsin and the edge with iron colloid staining. Thus, the top membrane contains free urobilinoid and urobilinoids bound to sugar derivatives loosely conjugated to proteins or mucoproteins, respectively.

II. *Subsequent separation of the urinary urobilinoid fractions by paper electrophoresis*

1. It is known that $CHCl_3$ urine extracts having the same R_f values as crystalline stercobilin in the two solvent systems mentioned contain stercobilin and i-urobilin (LEMBERG, LEGGE, 1949 d.) and in some cases d-urobilin (GRAY, 1953 b., WATSON, 1956, 1957), as well as their chromogens. According to our investigations these components show, on submitting them to paper electrophoresis in barbital buffer (pH 9,5) at 7 V/cm, a different mobility, but the difference of this mobility is not sufficient for their separation. All of them proceed towards the anode in the order of succession described above. I-urobilinogen (mesobilirubinogen) the only chromogen examined proceeds at the head. The electrophoretic behaviour of these free urobilinoids at varying pH-s and Volt numbers (KAHÁN, I. L. 1958) is discussed in chapter 4.

2. The fresh protein precipitate forming on shaking with ethylacetate or amylalcoholic $CHCl_3$ containing urobilinoid seems at first sight as if it would be an „oil in water" emulsion type. On standing, however, the stability of the emulsion decreases and simultaneously with colloid alterations the chromatographic behaviour of the urobilinoids of the ethylacetate protein emulsion or chloroform emulsion also changes. On washing the fresh emulsion with ethylacetate the substances obtained do not exhibit fluorescence after spraying with zinc acetate at chromatography in both solvent systems except at the site of application. On storing these emulsions for 24 hours at 4°C or the ethylacetate extracts of the fresh precipitates for an hour at 37°C, they only hardly show any fluorescence after spraying with zinc acetate at the

site of application, but a fluorescence area mainly corresponding to that of crystalline stercobilin can be demonstrated in most cases. On heating with anilin hydrogenphtalate no brown colour appeared at these sites. In spite of the fact that the fluorescence got weaker or disappeared at the start the protein staining with acid fuchsin and the mucopolysaccharide which may be developed with iron colloid staining, respectively, remained unchanged at the start. The urobilinoid component which migrates in a different manner to the crystalline stercobilin and can also be developed with anilin hydrogenphtalate could not be detected in chloroform or ethylacetate emulsion either on standing or after keeping the extracts at 37°C and not even after trypsin digestion of the emulsion on extraction with ethylacetate, although this conjugated urobilinoid is easily shaken into ethylacetate.

By the action of the electric field the urobilinoid splits off the protein too. On submitting the saturated solution of the chloroform or ethylacetate protein emulsion prepared with barbital buffer (pH 8,6) to paper electrophoresis for 5 hours fluorescence can be detected after spraying with zinc acetate at the site of the free urobilinoid pigments. With acid fuchsin proteins can be detected behind it quite until the start, but on the site of the urobilinoid already not. If staining is carried out with acid fuchsin it is deeper at the start and if it is performed with PAS and iron colloid it is deeper at the sites of the migrated protein fractions.

Thus on standing, incubation, or by the action of electric field these emulsions undergoe separation i. e. into proteins and mucoproteins and into free urobilinoid pigments behaving like crystalline stercobilin respectively.

The top mebrane forming on preparation with $(NH_4)_2SO_4$ butanol has already on first sight the appearance of a fatty lustrous reddish-brown emulsion of the „water in oil" type. Also without standing or incubation, at 37°C, the free urobilinoids and those conjugated to sugar derivatives split off partly on the action of the chromatographic solvent.

The substance moving in the same, or a different manner, as compared to stercobilin, can be washed off from the substance of the top membrane with ethylacetate or butanol. On incubating the top membrane for 16 hours in barbital buffer (pH 8,6) with 0,01 g/ml trypsin fluorescence is not visible at the start, but the two other urobilinoid components are still further detectable. From the barbital buffer (pH 8,6) solution of the top membrane the protein components split off after 5 hours paper electrophoresis. In the vicinity of the start they stain more distinctly with acid fuchsin and towards the anode with PAS and iron colloid staining procedures. One of the urobilinoids which split off can be found at the site corresponding to the free crystalline urobilinoid pigments. Another urobilinoid shows 1,4 fold mobility as compared to the crystalline stercobilin: this latter becomes brown on heating with anilinhydrogenphtalate reagent and the former not. — The top membrane obtained by preparation with $(NH_4)_2SO_4$ butanol can be separated by means of chromatography, washing with ethylacetate or butanol or with electrophoresis into proteins an or mucoprotein components and into free urobilinoid components as well as into those bound to sugar derivatives.

This latter protein-free component can be abundantly found in the upper oily layer obtained by the preparation according to Heikel. This phase also contains simultaneously free urobilinoids as reference substance hence for the examination of the component differing in motility from this crystalline urobilinoid the butanol supernatant was used. As on the basis of the development with aniline hydrogenphtalate besides urobilinoids, sugars or sugar derivatives could be suspected, this

butanol extract was submitted to electrophoresis in borate buffer (pH 8,6). The substance was separated into two urobilinoid bands. The spot localised 5 cm from the start showing a green fluorescence after spraying with zinc acetate corresponded to the position of the crystalline stercobilin. The second green fluorescing spot visible after zinc acetate spraying is 1,4 times so far as the former. (At the examination of fresh urines — containing the chromogens of the urobilinoids — green fluorescence appears in the immediate vicinity of the crystalline stercobilin spot. This has been discussed in detail in chapter 4.)

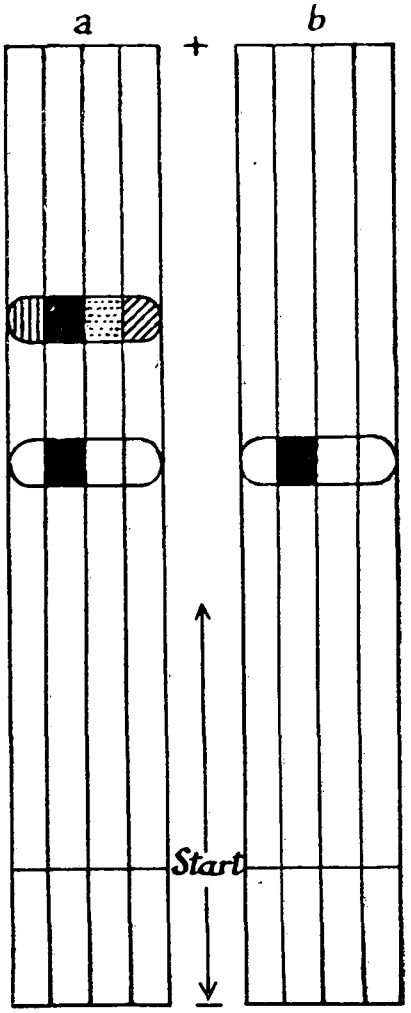


Fig. 10.
Paper electrophoretic pattern of $(NH_4)SO_4$ butanol extract of urine (a) for comparison (b) crystalline stercobilin pH 8,6; 0,25 M borate buffer
Schlesinger positive: ■
Anilin hydrogenphthalate: IIIII
Naphthoresorcinol: ⁞⁞⁞⁞
Morgan—Elson reaction positive: /////

If the spots of the substances with a 1,4 fold mobility compared to stercobilin are heated with anilin hydrogenphtalate they become brown whilst the substance migrating like crystalline stercobilin remains unchanged. The spots of a relative 1,4 fold mobility can in contrast to the free urobilinoid pigments be developed with naphtoresorcinol reagent giving a blue colour reaction characterising uronic acids. In the borate eluate of paper strips corresponding to the position of the spots of a relative 1,4 fold mobility compared to stercobilin the presence of uronic acid may be demonstrated with Dische's carbazol reaction too.

The spot of 1,4 fold relative mobility developed with the Morgan Elson reaction according to Partridge appears with a violet colour and this colour also develops on omitting acetylation with alkaline acetylaceton.

According to the above, the $(NH_4)_2SO_4$ butanol extracts of the urines contain besides free urobilinoid pigments urobilinoids containing sugar derivatives which move at a different rate as compared to the former substance if submitted to borate electrophoresis. The one having a 1,4 fold relative mobility is an urobilinoid pigment-uronic acid-acetylglucosamine compound. (Fig. 10.).

III. *Paper electrophoretic examination of the products forming by the action of β-glucuronidase* on conjugated urobilinoid fractions

On the basis of the naphtoresorcinol development and the carbazol reactions the suspicion arose that the urobilinoid fractions characteristed at borate electrophoresis by 1,4

fold mobility as compared to that of the free urobilinoid pigment — contain uronic acid, but this requires further evidence.

1. The effect of added β-glucuronidase on the carbohydrate bound urinary urobilinoid fractions

Half of the inactivated urine was incubated with Helix pomatia gastric juice containing glucuronidase or liver glucuronidase respectively, and the other half without these substances for 5½ and 20 hours, respectively. The borate electrophoresis fractions of the $(NH_4)_2SO_4$ butanol extracts of the urines treated with the two different glucuronidases showed essentially the same changes as compared to the untreated ones hence they will be described together (Fig. 11.).

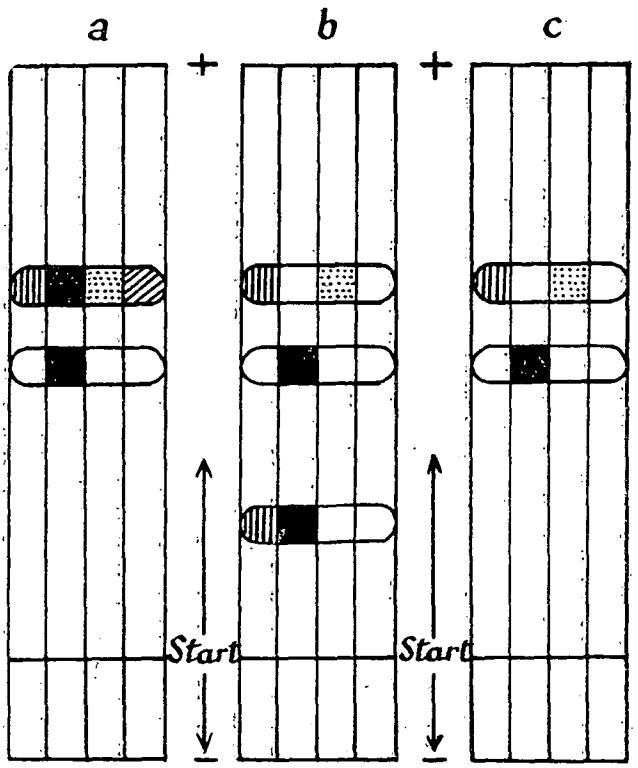


Fig. 11.

Action of β-glucuronidase added to inactivated urines upon the borate electrophoresis fractions of their $(NH_4)_2SO_4$-butanol extracts.

a) Extract of untreated urine
b) Extract of β-glucuronidase treated urine
c) Mixture of cryt. stercobilin and glucuronic acid
Schlesinger positive: ■
Aniline hydrogenphtalate: ||||
Naphthoresorcinol: ⁞⁞⁞
Morgan Elson reaction: ////

a) The urobilin-uronic acid-acetylglucosamine containing spot obtained after borate electrophoresis yields a positive reaction with Schlesinger reagent, naphtoresorcinol and Partridge-Morgan-Elson reagent, whereas the substance treated with β-glucuronidase gives a negative reaction with Schlesinger reagent and Partridge-Morgan-Elson reagent, but if developed with naphthorescorcinol an intensive blue colour appears at the corresponding site. Finally, if it is developed with aniline-hydrogenphtalate or formazan reaction the reducing capacity of β-glucuronidase-treated samples is greater than that of untreated samples.

According to the above observation on the action of β-glucuronidase urobilin- and acetylglucosamine split off from the fraction containing urobilin-uronic acid-acetylglucosamine and the glucuronic acid which also split off by the action of glucuronidase from the other urinary glucuronids remains on the site of the urobilin-uronic acid-acetylglucosamine fraction migrating with the same speed as the simultaneously run glucuronic acid in borate buffer (compared to dextrose 1,4 fold).

b) On the borate electrophoresis strip of the urine extracts treated with glucuronidase the free urobilinoids are visible on the same site, but more intensely than in the case of untreated urine.

c) On the action of β-glucuronidase a new fraction appeared on the borate electrophoresis strip of the urinary extract, i. e., between the start and the free urobilinoids. If the spot of this fraction is sprayed with Schlesinger reagent it shows a green fluorescence and according to the developments it may be established that it does not contain either uronic acid or glucosamine, but if it is developed with anilinhydrogenphtalate which indicates reducible sugars it becomes brown. In contrast to other conjugated urobilinoids this fraction like free urobilinoids can also be extracted with chloroform. When the eluate of this fraction is hydrolysed for an hour at 100°C with 5-N H_2SO_4 after neutralisation with $Ba(OH)_2$, mannose, glucose and galactose can be detected in the hydrolysate by means of descending chromatography in pyridin-butanol-water. Hence, on the action of glucuronidase urobilinoid is released part of which is bound secondarily to the neutral oligosaccharides composed of these sugars. (It should be noted that on the one hand, the decrease or disappearance of the fraction containing uronic acid, and on the other the increase of the free urobilinoids, as well as at the appearance of the fraction which shows a brown colour with aniline-hydrogenphtalate and migrates between the start and the free urobilinoids when subjected to borate electrophoresis, could be observed in some cases where urine samples had been left standing without glucuronidase but not heatinactivated.)

2. For the other change induced by the action of the glucuronidase added to the inactivated urine, i. e. for the formation of the fraction which can be detected by borate electrophoresis at the site between the start and the free urobilinoid, the presence of another substance of the urine, i. e. the oligosaccharide containing mannose, glucose and galactose is required, to which the urobilinoid pigment released by the glucuronidase is secondarily bound. The following observation refers to the origin of this oligosaccharide:

On incubating half of the saturated solution of urinary mucoprotein with crystalline stercobilin for an hour and the other half with crystalline stercobilin and liver β-glucoronidase the $(NH_4)_2SO_4$ butanol extract of both reaction mixtures were examined by means of borate electrophoresis. As control the crystalline stercobilin extract incubated with liver β-glucuronidase was used (Fig. 12.).

On the electrophoresis paper strip of the urinary mucoprotein incubated with β-glucuronidase and stercobilin a new urobilinoid spot was visible between the start and the well detectable free stercobilin spot showing a green fluorescence after spraying with Schlesinger reagent and showing a brown colour after heating with aniline-hydrogenphthalate. The mucoprotein incubated with stercobilin, but without β-glucoronidase and the β-glucuronidase extract only incubated with stercobilin, only showed fluorescence at the site of the free urobilinoids, but not between this site and the start. It should be noted that on carrying out a control with protein staining, the protein of the β-glucuronidase preparation can be demonstrated near the site corresponding to that of the free urobilinoids, but on the site of the band of the urobilinoid conjugated to the new oligosaccharide protein staining was absent.

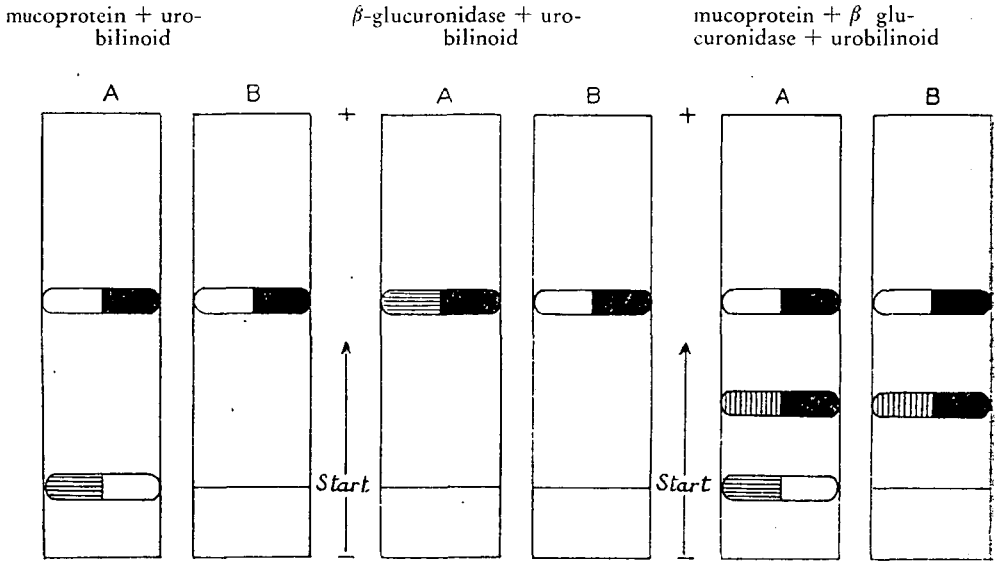


Fig. 12.
Incubation of stercobilin with urinary mucoprotein and β-glucuronidase.
A: $(NH_4)_2 SO_4$-butanol extract. B: $CHCl_3$-extract.

Schlesinger positive:
Aniline hydrogenphtalate:
Stained with protein stain:

Hence on the action of β-glucuronidase from one of the isolated mucoproteins of the urine an oligosaccharide capable to bind secondarily to the released urobilinoid pigment forms. The urobilinoid migrates together with this oligosaccharide in the course of borate electrophoresis at the site between the start and the free urobilinoid, whereas the untreated mucoprotein or the β-glucuronidase alone do not form such an electrophoretic fraction with crystalline stercobilin.

Conclusions

I. The qualitative examination of the urobilinoids present in comparatively high dilution (0,1 mg—50 mg/l) in normal or even pathologic urines containing many other disturbing substances is only possible if previously some extraction procedure is

applied. Thus it is indispensable to examine whether some isolated urobilinoid fractions are not artifacts due to extraction.

Most of the urobilinoids are secondarily conjugated to the emulsion containing protein or mucoprotein, respectively, in the course of the extraction procedures. This is supported by the following:

a) The native protein or mucoprotein respectively, is only present in very small quantities in the untreated urine.

b) According to the extraction procedure applied, different urobilinoids are conjugated to the protein emulsion, i. e. on shaking with amylalcoholic $CHCl_3$ or with ethylacetate free urobilinoid pigment and on extracting with $(NH_4)_2SO_4$ butanol in addition a great quantity of urobilinogen-uronic acid-acetylglucosamine is bound.

c) As long as the emulsion is stable urobilinoid is strongly bound to the $CHCl_3$ amylalcoholic or ethylacetate precipitate; as soon as the dispersity decreases the urobilinoid easily splits off from the emulsion.

d) In every case the ability to wash off the urobilinoid with an organic solvent depends upon the fact whether the solvent itself forms the dispergent medium. In the butylalcoholic top membrane the butylalcohol is on the surface of the protein drops and the urobilinoid may be washed out of them with butylalcohol, whereas in the case of the fresh $CHCl_3$ amylalcoholic emulsion the $CHCl_3$ drops are kept away by an aqueous protein coating from the organic solvent. At the change of the emulsion the urobilinoid can already be washed out.

All this concerns the great quantities of urobilinoids adsorbed by the great surface of emulsions formed by small amount of denaturated urinary protein. Urobilinoids are conjugated to serum proteins also in their native state (cf. Chpter 7.).

The literature reports about conjugation of faecal stercobilin to pyrogen mucopolysaccharides (Watson, 1948—49/a).

II. The changes occurring on the action of β-glucuronidase render it possible to draw conclusions concerning the nature of the urobilinoid compounds excreted with the urine.

The fact that the urobilin-uronic acid -acetylglucoseamine decomposes on the action of β-glucuronidase added to the inactivated urine proves that this fraction contains uronic acid, i. e. glucuronic acid.

The β-glucuronidase of the urine plays a double role in producing the urobilinoid fraction bound to the oligosaccharide appearing also in the course of standing and migrating characteristically during borate electrophoresis between the start and the free urobilinoids:

a) it releases urobilinoid which being oxidised to urobilinoid pigment is capable to be bound to the oligosaccharide containing mannose-glucose-galactose; b) it forms by splitting the mucoproteins of urine oligosacharides able to conjugate urobilinoid pigments.

As free urobilinoids and the urobilinoid pigments conjugated to the above oligosaccharides are chloroform soluble — as contrasted to the form esterified with glucuronic acid — it is comprehensive that on the action of β-glucuronidase the extraction with chloroform becomes more complete.

7. UROBILINOIDS IN THE BLOOD

Already as far back as in 1923 BARRENSCHEEN dealt with urobilinoids of the blood serum. By means of qualitative and quantitative tests WINTERNITZ (1925) and ROYER (1928) established that if normal serum does contain urobilinoid it can only be present in quite insignificant amounts. According to some authors the urobilinoids of the serum are not conjugated to proteins and the low serum urobilinoid level is due to this (FARMERLOEB 1932). WITH discusses this question in detail in his book entitled „The Biology of the Bile Pigments" and he advances the view that the urobilinoids of the serum are not linked to the proteins (WITH, 1954). Recent literary data on this subject were also referred by him, he also states that porphyrins like urobilinoids are not conjugated to proteins (WITH, 1958).

The problem of the serum urobilinoids has again gained up-to-dateness owing on the one hand, to the recognition of the fact that bilirubin — the reduction products of which are the urobilinoids — is bound to proteins and on the other, because the linkage of the urobilinoids to other proteins, i. e. urinary proteins and the muco-proteins of the intestine has been observed (s. Chapters 6 and 8). Thus the following investigations were carried out:

A) To human serum crystalline urobilinoid (stercobilin) was added in vitro and its conjugation to serum protein studied.

B) Crystalline urobilinoid (stercobilin) was added in vitro to dog serum and its conjugation to the proteins of dog serum examined.

C) The conjugation of the urobilinoids to serum protein following absorption from an isolated intestinal segment was investigated by application of stercobilin in dogs.

Methods

A) 1. 10 μg of stercobilin dissolved in 0,05 ml saline was added to 1 ml of serum of a healthy individual and incubated for an hour at + 4°C. 0,05 ml of this serum was submitted to paper electrophoresis.

2. 0,5 ml of the serum of a healthy person was diluted 1:1 with phys. NaCl and subsequently incubated with 5 μg stercobilin in the same way as indicated above.

3. Electrophoretic separation was carried out on 4×40 cm horizontally placed Schleicher Schüll 2043 b paper strips at 4 V/cm voltage in borate buffer at pH 8,6 for 2, 4, 6 and 8 hours.

4. The developments were performed in the case of urobilinoids with Schlesinger reagent (SCHLESINGER 1903), in that of proteins with acid fuchsin (NOVOTNY 1952), according to the modification of LAURELL and SKOG (1956). Hydrochloric acid

hydrolysis wos accomplished (100 ml ethylalcohol +3 ml conc. HCl) for 5 minutes at 100°C.

B) According to A/1 stercobilin was added to the serum of a healthy dog and the electrophoresis as well as the development was carried out according to A/3 and A/4 respectively. To dog blood withdrawn into heparinized syringes stercobilin was added similarly to A/1.

C) 1. Into an isolated segment of the small intestine of a dog 6 mg of stercobilin* dissolved in 15 ml saline was introduced (the solution was prepared with water

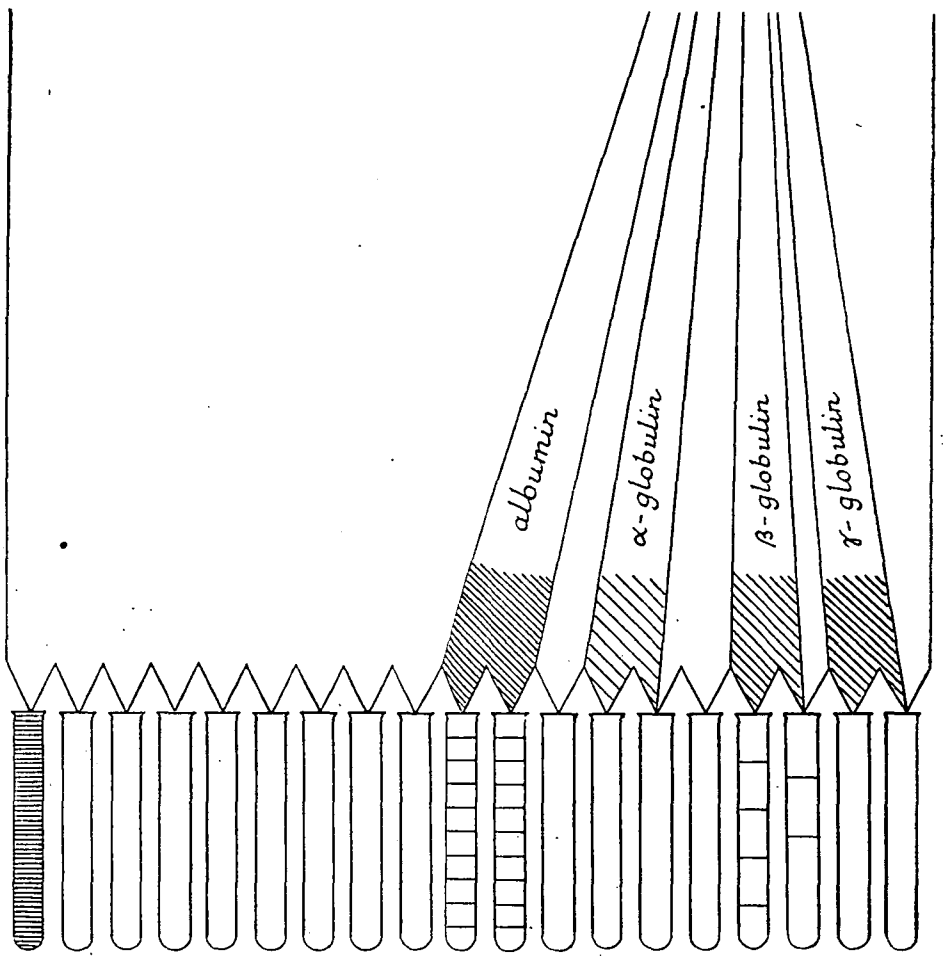


Fig. 13.
Scheme showing the fluorescence of plasma urobilinoid fractions obtained by continuous electrophoresis. The extent of the fluorescence recorded by means of the instrument is indicated by the density of the lines of the tubes.

* I am fully aware of the fact of having dealt with urobilinoid pigments similar to, but not identical with the urobilinoids of the intestine, their chromogens.

distilled from potassium permanganate and sodium hydroxide and tested for fluorescent impurities) in chloralose-anesthesy. Every three minutes blood was withdrawn from the mesenteric vein the coagulability of which had already been abolished at the beginning of the experiment by administration of heparin.

2. The urobilinoid content of the plasma obtained after centrifugation was measured by means of a fluorometer (FAREDIN—SÁRKÁNY, 1958) (s. Chapter 8).

3. The plasma showing the greatest fluorescence was submitted to continuous electrophoresis. A voltage of 350 V (10 V/cm) was applied and 1 ml was fractionated for 10 hours at pH 8,6 in Michaelis barbital buffer with an ionic strength of 0,1.

As can be seen in Fig. 13 fractions were collected into glass vessels. After the electrophoresis the paper was stained with amidoblack 10 B according to Novotny (1954). The fractions collected in the vessels diluted with buffer in the course of the electrophoresis were examined under an analytical quartz lamp. The fractions exhibiting a visible fluorescence as well as those which contain according to the protein staining of the electrophoretic pattern the albumin and globulin fractions were submitted to fluorescence examinations. For this purpose the solutions were adjusted to pH 7,0 and subsequently filled up to identical volumes (12 ml) with fractions which ran down between the γ-globulin and the cathodes (with a protein and urobilinoid free buffer solution). For blanks the same buffer solution was used. Then the fluorescence was examined. (Owing to the zinc traces present in the serum it was not necessary to add Zincacetate for the formation of an urobilinoid Zn complex.)

Results

A) Urobilinoid binding capacity of human serum proteins in vitro.

As seen on Table 4 urobilinoid is readily conjugated to the proteins of the serum. It is completely bound to undiluted normal serum proteins, free urobilinoid only splitting off after prolonged electrophoresis (8 hours). Urobilinoid added to diluted serum partly splits off the proteins already after 4 hours electrophoretic run and migrates as free unconjugated urobilinoid. Among the serum proteins urobilinoid is mainly bound to albumin and this linkage is the most stable. Conjugation to β-globulin could also be found, but it proved to be less stable in serum diluted with saline, and fluorescence could not be demonstrated at the β-globulin area. Presumably

Table 4.

Time points when stercobilin fluorescence of serum proteins could be detected on electrophoretic paper strips by means of the Schlesinger reagent.

Serum component	Undiluted serum			Serum diluted 1:1 with 0,9% NaCl solution		
	4h.	8h.	20h.	4h.	8h.	20h.
Albumin	+	+	+	+	+	
α-globulin						
β-globulin	+	+	+			
γ-globulin						
free stercobilin		+		+	+	+

this loose linkage breaks down in the course of the electrophoresis. Other urobilin fractions conjugated to proteins could not be found. The proteins exert a certain stabilising effect becoming obvious in the present experiment by the fact that after 20 hours of electrophoresis only that part of the urobilinoids, added to the undiluted serum, decomposes, which was separated from the protein during the electrophoresis. There was no free urobilinoid present, and the urobilinoids bound to the albumin and β-globulin could be detected on the electrophoresis paper strip. Moreover only the small amount of the urobilinoid added to diluted serum could be determined which just split off from the last protein conjugation.

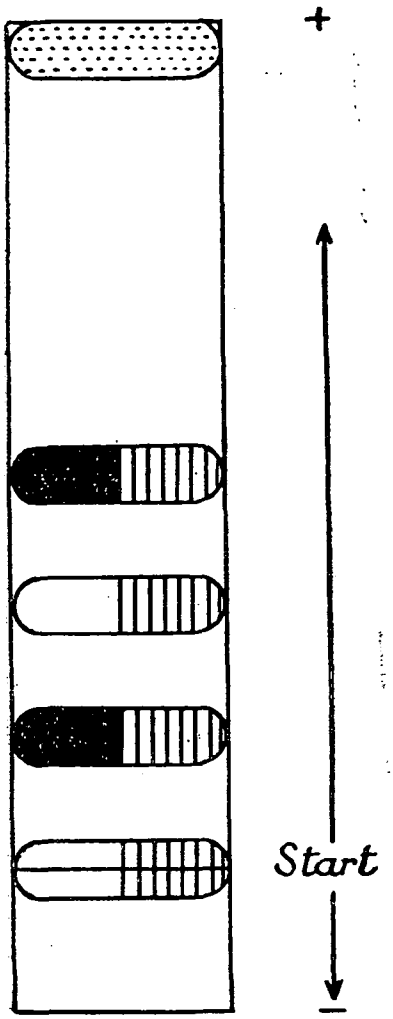


Fig. 14.
Electrophoretic pattern of heparinized canine blood plasma.
Schlesinger positive:
Schlesinger positive following hydrolysis with hydrochloric acid:
Stained by protein dyes:

B) Urobilinoid binding capacity of canine serum proteins in vitro.

B) 1. On carrying out the experiment similarly to A) we succeeded in detecting urobilinoid conjugated to albumin and β-globulin. That the urobilinoid migrates after all in a protein linkage and not as free urobilinoid is also shown by the stabilising effect of the protein conjugation. Urobilinoid incubated with dog serum and applied to electrophoretic run for 20 hours is well visible even to the naked eye by its yellow colour — at the site of albumin — whereas the same quantity of urobilinoid dissolved in saline cannot be demonstrated even by the sensitive zinc complex fluorescence reaction after a similar electrophoretic run, i. e. it has completely decomposed.

2. The electrophoretic pattern of dog blood plasma withdrawn into heparinized syringes showed in addition to the urobilinoid bound to albumin a distinct reddish band which took place at double the distance from start when referred to albumin. The latter, however, was not fluorescent after spraying with Schlesinger reagent: it only showed this phenomenon after hydrolysis with hot alcohol containing hydrochloric acid, whereas that bound to albumin and β-globulin gave the usual green urobilinoid fluorescence spontaneously after spraying. (Fig. 14.). If before the electrophoresis heparin was added to the urobilinoid, fluorescence with Zn ions could not be produced and in the course of the electrophoretic migration the total amount of urobilinoid appeared as a red band at the

double the distance from the application as compared to albumin in the position mentioned above. This observation points to the fact that a conjugation can form between the urobilinoid and heparin.

C) Binding of resorbed urobilinoids by canine plasma proteins and heparin.

The data obtained with the continuous electrophoretic apparatus are illustrated in Fig. 13. (Owing to the small number of examinations the results are only regarded as semiquantitative data.) It can be seen in the figure that according to the fluorescence data about half of the urobilinoids resorbed into the blood can be found in a fraction dropping in the course of the electrophoresis at about double the distance from the application as compared to the albumin. The urobilinoid which gained access besides this into the blood migrates bound to proteins, mostly to albumin and to a small extent to β-globulin.

Conclusions

As can be seen from the experimental results the urobilinoids are readily conjugated to serum proteins and their behaviour also resembles that of their mother compounds e. g. bilirubin. Many authors have described the conjugation of bilirubin to albumin (BENNHOLD, 1932; GRAY, KECKWICK, 1948; NAJJAR, 1952) and some its linkage to α-globulin (MARTIN, 1948). KLATSKIN and BUNGARDS (1956) report that it is bound to albumin and according to them if there exists a conjugation between α-globulin and bilirubin it is not appreciable. The literature also mentions its linkage to β-globulin (BENNHOLD, 1953). This occurs according to Bennhold if bilirubin is in excess, i. e. in addition to the albumin and α-globulin linkage the residual bilirubin can be linked to β-globulin. That an urobilinoid linkage to α-globulin cannot be observed is probably due to the fact that this conjugation is less strong than the albumin one, hence if the electrophoresis is prolonged the urobilinoid splits and decomposes, whereas in the case of a short-time electrophoresis the separation is not complete.

As mentioned above proteins exert a stabilising effect on urobilinoids. This may be explained by their protective effect against oxidation occurring during the electrophoresis.

Finally the heparin bound urobilinoid fraction migrating at a very great rate occuring when it is withdrawn into heparinized syringes must still be mentioned. This observation suggests that a conjugation between the molecules containing urobilinoids and other acid groups forms readily. Curiously enough if the urobilin is bound to a molecule containing a strongly acidic group it looses its fluorescence and a red colour appears. The literature contains the description of a similar phenomenon (FISCHER, REINECKE, 1939).

8. FORMATION OF UROBILINOIDS

I. Site of urobilinoid formation

It is known, that within the reticuloendothelial system (RES) the bile pigments containing a straight tetrapyrrol chain form through oxidative opening at the methene bridge of the porphyrin ring from the haemoglobin of the destroyed erythrocytes. At the conversion first of all biliverdin forms, but through rapid reduction it is still converted within the cells of the RES to bilirubin. From the bilirubin via the less important mesobilirubin in which the bilirubin vinyl groups have already been reduced to ethyl groups, urobilinoids are formed (in a wider sense their chromogens also can be ranged here).

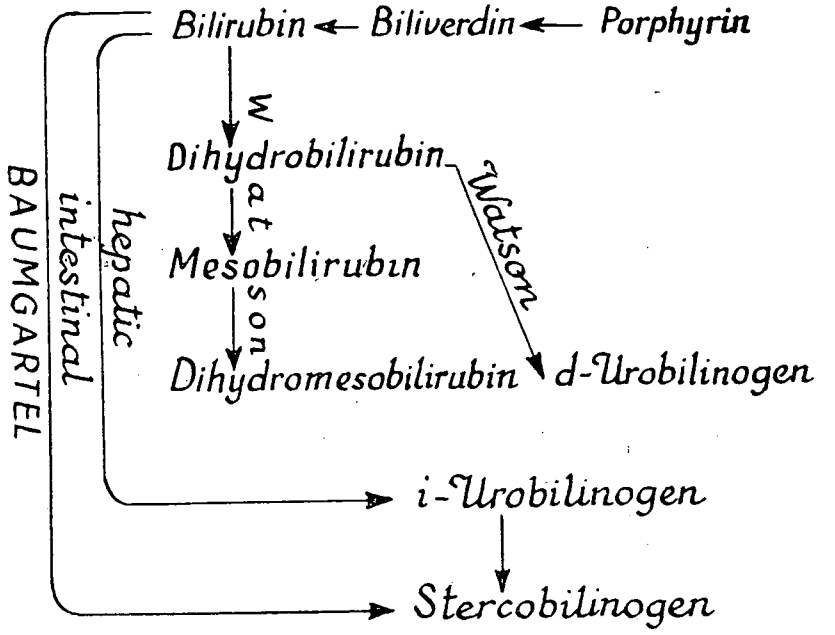
Concerning the site at which urobilinoids form previously two concepts were elaborated: the theory of the enteral and that of the hepatogen formation. The former was proved by the experiments of FR. V. MÜLLER (1892), the latter by FISCHLER (1908).

The establishment that urobilinoids are not uniform (WATSON 1934, LEMBERG 1934, demonstrated that urobilin and stercobilin are two different compounds) gave rise to a dual theory the representatives of which are BAUMGARTEL (1943) and his school. According to BAUMGARTEL (1943) stercobilinogen forms as a result of bacterial reduction in the gut and i-urobilinogen as that of enzymatic reduction in the liver cells (BAUMGARTEL, 1949 a., b.), both are directly reduced from bilirubin. He suggests that the reduction in the gut takes place as follows: the anaerob Bac. verrucosus reduces the cystin of the bile in the course of the formation of cystein in the coecum. A dehydrase system forms: cystein $\underset{-H_2}{\overset{+H_2}{\rightleftharpoons}}$ cystin, in which the dehydrogenase of Bacterium coli plays the role of a catalyst which mediates the hydrogen of the cystein to the hydrogen acceptor. Bilirubin is such a hydrogen acceptor which is reduced owing to the hydrogen uptake.

Whereas according to BAUMGARTEL owing to the specific action of the bacterial dehydrase of coli the reduction can only proceed in the direction of stercobilinogen the liverpulp particularly in the presence of cystin reduces biliverdin to bilirubin and then to i-urobilinogen (mesobilirubinogen). In view of the fact that the liver always contains cystein BAUMGARTEL believes that also in this case the cellular reduction is in relation with the conversion of cystin to cystein. Hence, according to him the enterogen reduction process through which stercobilinogen forms from bilirubin can be strictly separated from the cellular enzymatic process by which i-urobilinogen becomes the end product of the bilirubin reduction. The bilirubin urobilinogen reduction process only takes place in the case of impairment of the liver — and in that of biliary obstruction associated with liver parenchyme damage.

The theory of BAUMGARTEL is contradicted by the fact that when there is obstruction of the bile, although impairment of the liver also prevails, the excretion of i-urobilinogen ceases too. BAUMGARTEL attributes this to the blocking effect exerted by bilirubin in the reducting enzymes (BAUMGARTEL, 1952, 1958).

In addition to the above, numerous earlier experiments, as well as the results of therapeutic and laboratory investigations carried out with modern devices of WATSON and his school, contradict the theory of BAUMGARTEL.



1. WATSON has proved experimentally that i-urobilinogen (mesobilirubinogen) excretion must not be associated with damage of the liver, but its occurrence depends upon the intestinal flora. If patients suffering from haemolytic icterus are given terramycin or aureomycin the faeces becomes completely urobilinoid free and instead of it only bilirubin can be detected. After a certain time following the withdrawal of the administration of antibiotics first d-urobilinogen excretion could be observed (WATSON, 1956; SBOROV et al, 1951).

2. The second decisive experiment of Watson, the in vitro bacterial reduction of mesobilirubinogen (i-urobilinogen) to stercobilinogen disproved the dual theory of BAUMGARTEL (WATSON, et el, 1942; WATSON, et al, 1954).

The experiment was later also carried out with i-urobilinogen containing N^{15}. The procedure to obtain N^{15} labelled i-urobilinogen was solved wittily: A patient suffering from haemolytic icterus was fed with N^{15} labelled glycin, after being administered terramycin he excreted N^{15} labelled bilirubin in large amounts. This was chemically reducted to i-urobilinogen and the substance thus obtained was submitted to bacterial reduction in vitro (LOWRY, et al, 1954).

3. The reduction by chemical means of d-urobilinogen to i-urobilinogen proves together with the above experiment that all three urobilinoids can be converted into

one another by successive reduction (LOWRY, et al, 1952). The most recent investigations of WATSON (1959) confirm that d-urobilinogen is also excreted under normal conditions, i. e. d-urobilinogen also forms in the course of normal bacterial reduction.

By means of urinary i-urobilin and stercobilin determinations it was attempted to make use of the Baumgärtel theory for clinical diagnostic purposes to differenciate hepatocellular and haemolytic icterus. Such examinations were carried out by STICH (1948), under normal conditions and in the case of haemolytic icterus he only found stercobilin whereas with hepatitis he detected i-urobilin in the urine. Our examinations

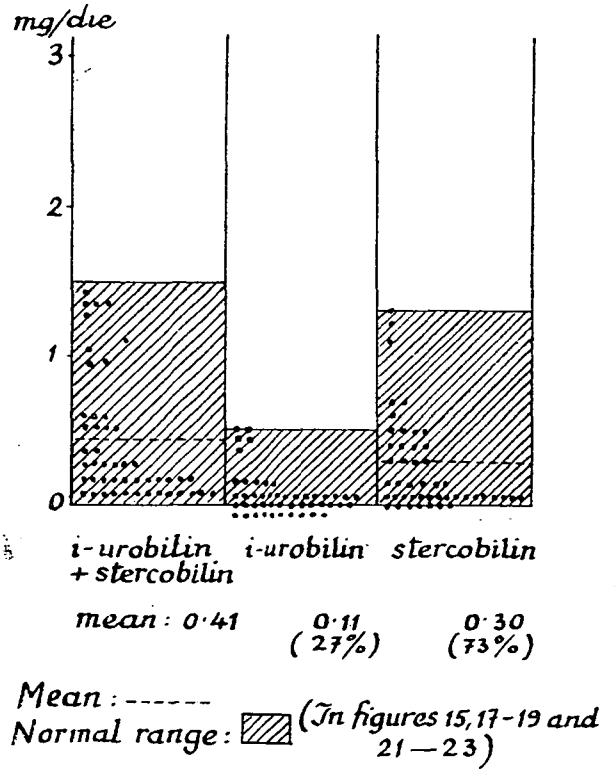


Fig. 15.
Daily urinary output of i-urobilin and stercobilin. 47 urine samples of 26 normal individuals were analysed.

(KAHÁN, I. L., FELKAI) described below could not confirm the results of BAUMGÄRTEL and STICH.*

Fig. 15 shows the results of 47 urinary urobilinoid examinations of 26 healthy individuals. On the basis of these examinations the normal values are as follows: total urobilinoid 1,5 mg/die. Regarding its components: i-urobilin 0,5 mg/die,

* The determinations were carried out according to the procedure described in chapter 5.

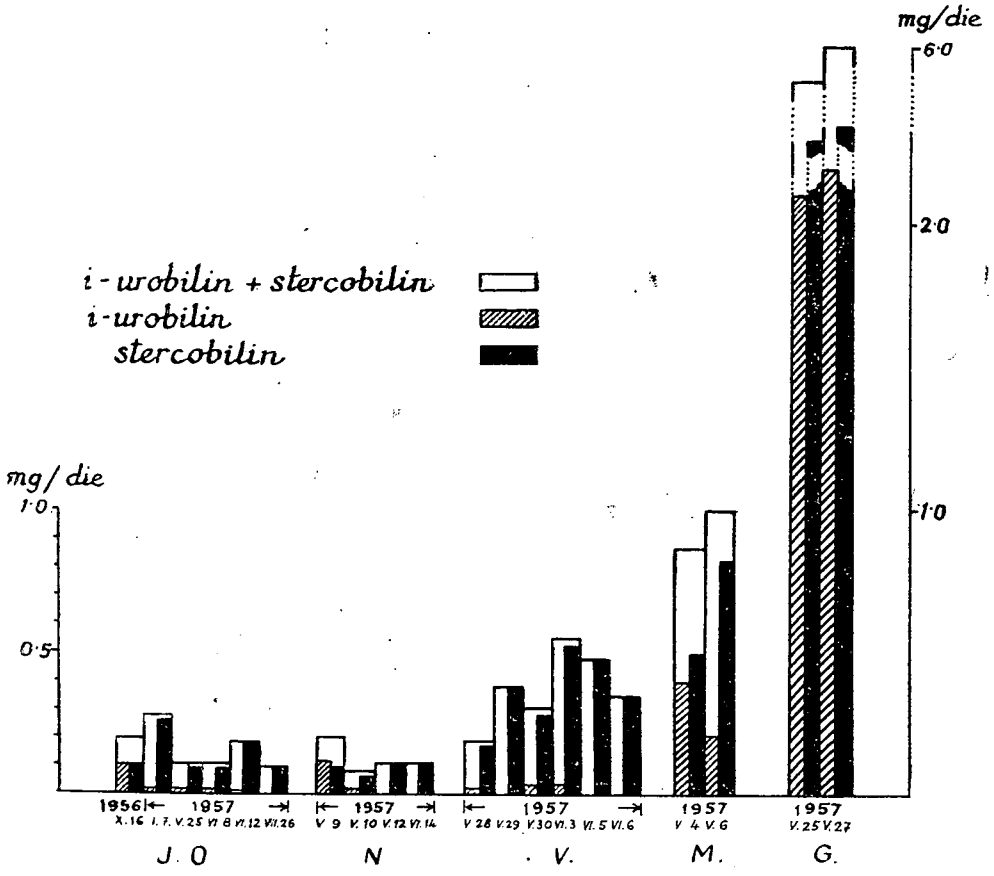


Fig. 16.

Daily urinary i-urobilin and stercobilin excretion of 4 different normal individuals (J. O., N., V., M.) measured on different days. — For comparison output of one patient suffering from cirrhosis hepatis (G.).

stercobilin not exceeding 1,3 mg/die. This figure and Fig. 16 demonstrate that the urinary urobilinoid output of healthy persons mainly consists of stercobilinogen, but i-urobilinogen can also be found. In the latter figure it seems striking that within the limits of the relatively low output of healthy persons, in some urine samples comparatively high, in others relatively low urobilinoid excretion can be found.

In the field of clinical pathology investigations were carried out in different types of obstructive jaundice (Fig. 17). A low total urobilinoid excretion was found (0,37 mg/die) and within this the stercobilin value was also strikingly low (0,16 mg/die). Thus with obstructive jaundice stercobilin only amounts to 35 per cent of the total urobilinoids, whereas normally it is 73 per cent.

For the investigation of hepatocellular jaundice the urine of patients suffering from cirrhosis hepatis and hepatitis epidemica was used. It can be seen in Fig 18 that the total urobilinoid output of patients with cirrhosis hepatis may increase to a great extent (maximally 18,6 mg was measured) but in many cases it is normal. Stercobilin amounts to 61 per cent of the total urobilinoids.

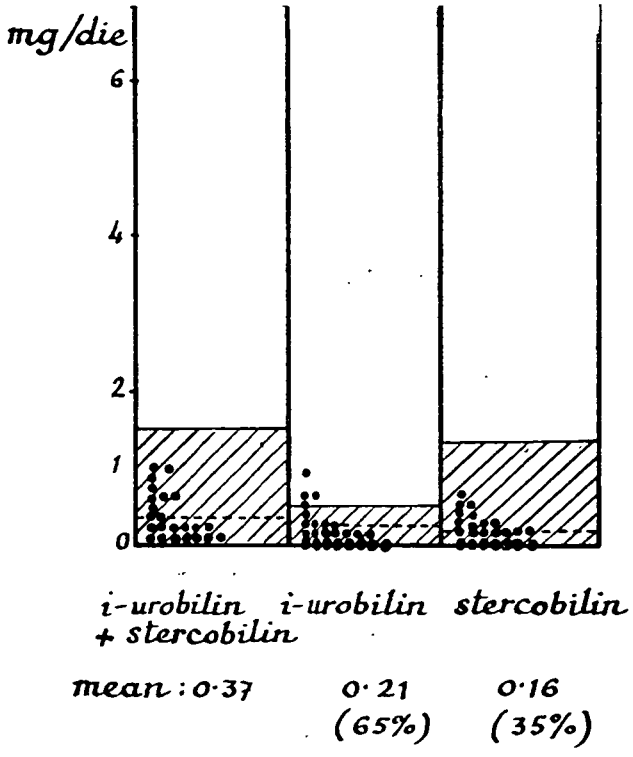


Fig. 17.

Daily urinary i-urobilin and stercobilin values from patients suffering from bile tract occlusion (stone, tumour). 24 urine samples from 11 patients were analysed.

Fig. 19 and 20 exhibit the results of the investigations concerning hepatitis epidemica. It may be seen that in the case of hepatitis epidemica i-urobilin and stercobilin both increase considerably in the urine. Fig. 20 illustrates that in the course of the hepatitis the urobilinoid excretion changes according to the different phases. In the intrahepatic obstructive phase (bile pigment does not reach the intestinal tract) the total urobilinoid excretion decreases to a great extent. When the obstruction ceases it increases significantly, and from the urobilinoid components stercobilin dominates.

Figs. 21 and 22 show that with haemolytic jaundice the i-urobilin and stercobilin content of the urine both may increase.

These examinations also demonstrate that the ratio of the i-urobilinogen and stercobilinogen output respectively, does not behave characteristically in the various types of icterus. In the case of haemolytic jaundice not only the enhancement of the excretion of stercobilinogen, but also that of i-urobilinogen can be detected and with hepatocellular jaundice not only the i-urobilinogen excretion is enhanced, but that of stercobilinogen is also higher. Thus *for the differenciation of these two kinds of jaundices the separate determination of i-urobilin and stercobilin does not seem suitable.*

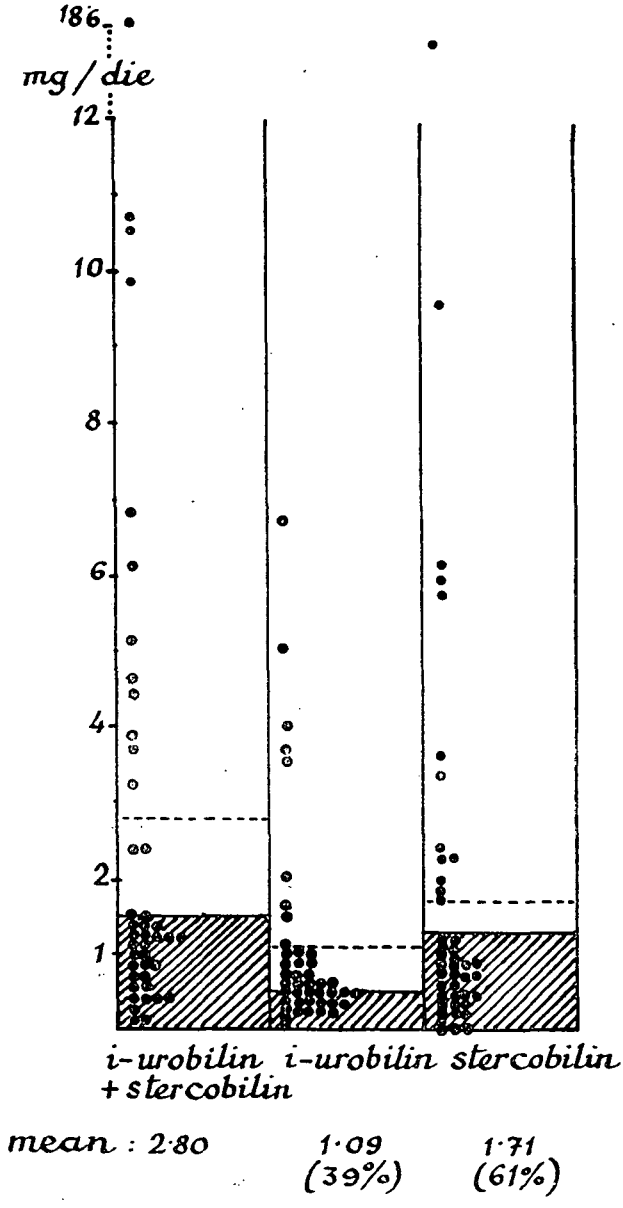


Fig. 18.
Daily urinary i-urobilin and stercobilin values from patients suffering from cirrhosis hepatis. 43 urine samples of 13 patients were analysed.

Our investigations suggest that the extent of the urobilinoid excretion does not depend upon the type of the icterus, but upon the fact whether or not the bile can get freely access into the intestinal tract, i. e. our results support the enteral theory. The suggestions mentioned above are also supported by the fact that urobilinoid output of patients suffering from obstructive jaundice is still lower than the normal one also in cases when the serum bilirubin level is not very high and the enzyme inhibiting effect of bilirubin suggested by BAUMGARTEL cannot come into play.

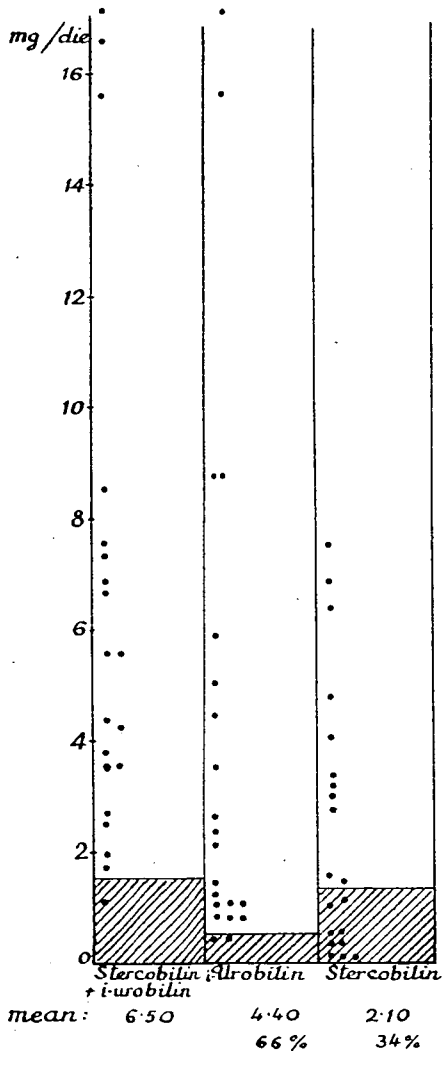


Fig. 19.
Highest urobilinoid output values of 20 patients suffering from hepatitis epidemica.

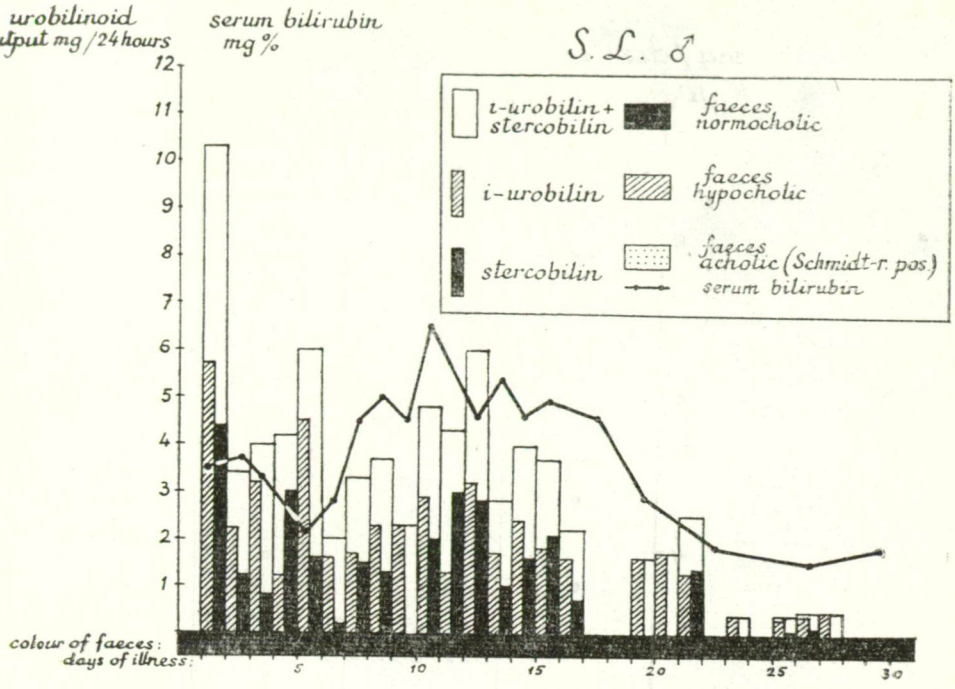


Fig. 20.

Daily urobilinoid determinations of one patient suffering from hepatitis epidemica.

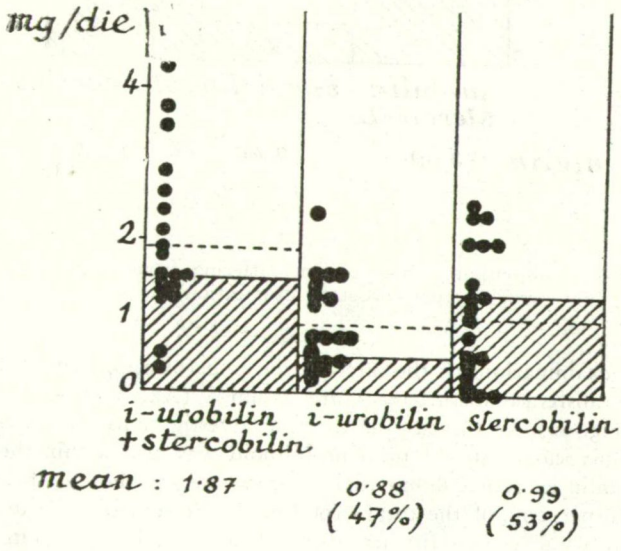


Fig. 21.

Daily urinary urobilinoid output from patients suffering from congenital haemolysis. 20 urine camples of 4 patients were analysed

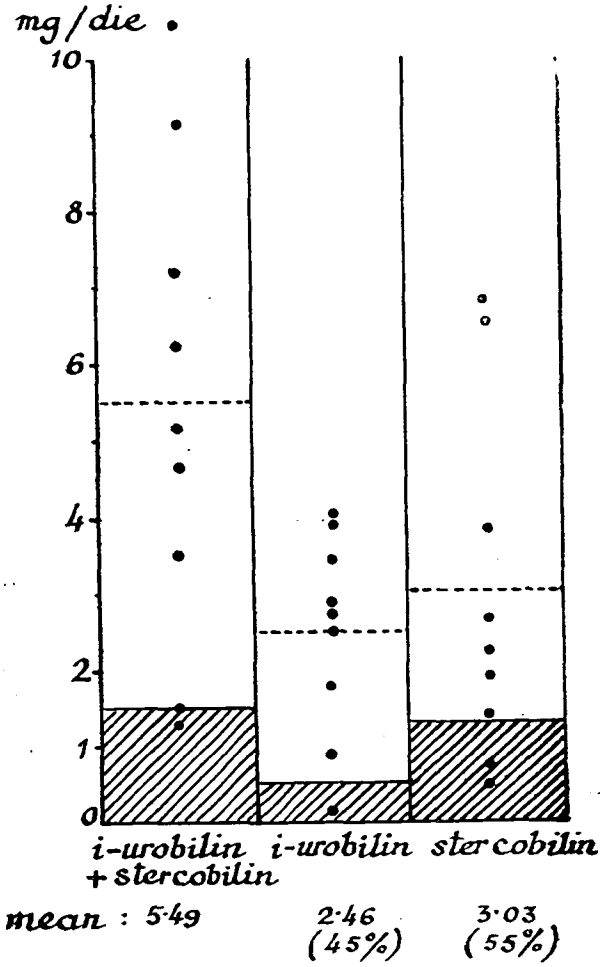


Fig. 22.

Daily urinary urobilinoid output of one patient suffering from aquired haemolytic jaundice (uranylacetate intoxication).

Finally the examinations of the intermitting hyperbilirubinaemia cases — in which glucuronicacid transferase disturbances are assumed (ARIAS et al., 1957) — should be mentioned (Fig. 23). In the case of the few investigations which, however, always showed an unequivocal result the total urobilinoid was also within the normal values. Although stercobilin excretion dominated yet compared to those of healthy individulas it was lower (60 per cent of the total urobilinoid). Presumably the disturbance of the bilirubin esterification causes further disturbances in the bilirubin decomposition, namely the reductive degradation of the free bilirubin becomes incomplete and the urobilinoid reduction like that of the inhibited reduction of the free bilirubin (WATSON, 1958) also takes place restrictedly.

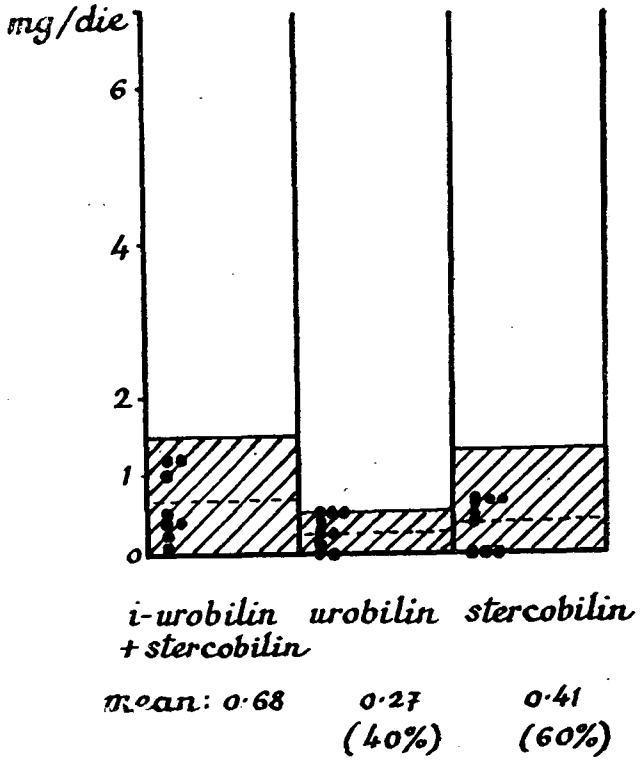


Fig. 23.
Daily urinary urobilinoid output of patients suffering from intermittent hyperbilirubinemia. 8 urine samples of 4 patients were analysed.

II. Resorption of urobilinoids

In the first part of this chapter results of investigations supporting the theory of enterogenous formation of urobilinoids are reported. The examinations described below are aimed at following up the fate of the urobilinoids formed in the intestine. For this purpose stercobilin dissolved in saline was introduced into the isolated small intestine segment of a dog and A) the intestinal juice sucked off at the end of the experiment, B) the venous blood withdrawn from the corresponding intestine portion, and C) the urine, were examined.

Methods

1. Stercobilin was obtained according to the method of FISCHER et al. (1939) from the urine of a patient suffering from haemolytic jaundice.

2. The urobilinoids were obtained from the intestinal content and the urine by the method described in chapter 5 by means of extraction with ammonium sulphate-butanol.

3. Ascending chromatography was carried out on Whatman No. 3 paper with chloroform : butanol : pyridine solvent as described in chapter 5.

4. Paper electrophoresis was performed in borate buffer in the manner recorded in chapter 6.

5. The developments for urobilinoids, urobilinogen, protein and mucoprotein were accomplished as described in chapter 5. The hydrochloric acid hydrolysis was performed by the spraying of the chromatograms and paper electrophoretic strips with hydrochloric acid alcohol (100 ml ethyl alcohol + 3 ml conc. HCL; heating 5 minutes at 100°C).

6. The measuring of the urobilinoid concentration of the blood plasma was performed by measuring the urobilinoid zinc fluorescence with the Faredin-Sárkány

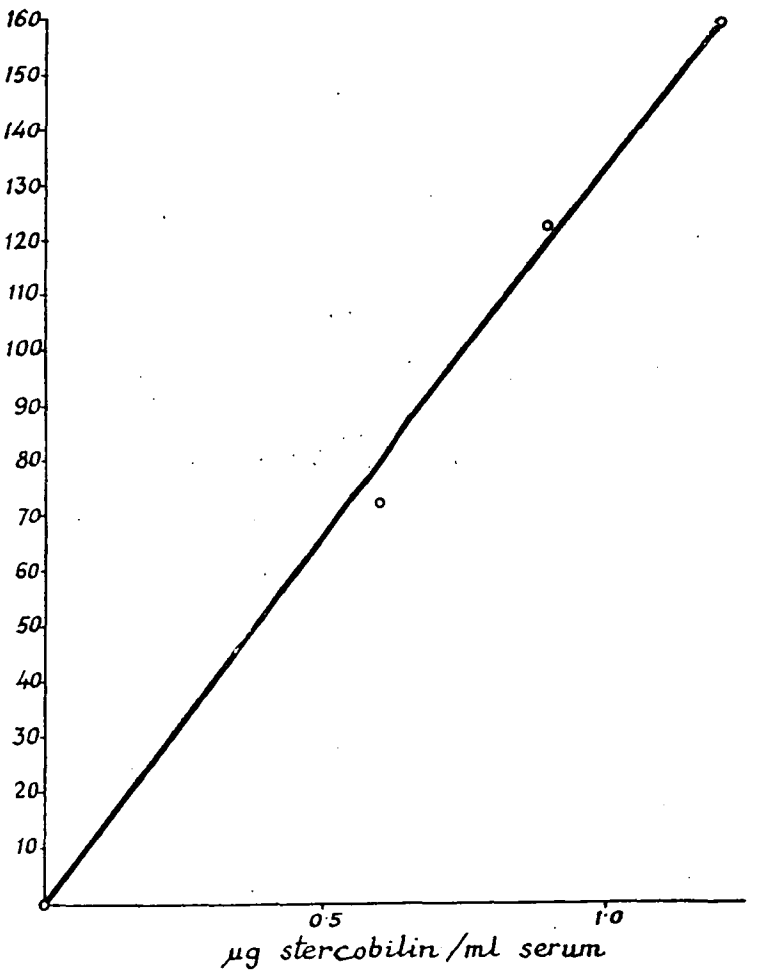


Fig. 24.

Calibration curve for serum urobilinoid estimation. The amount of crystalline stercobilin added to the serum is plotted on the abscissa. The extent of fluorescence is shown on the ordinate. Excitation light emission maximum at 473 mμ. Fluorescence light measured with filter having an absorption maximum of 533 mμ. Fluorescence light measured with a galvanometer having a sensitiveness of 5×10^{-9} A/ scale range.

fluorometer (FAREDIN et al, 1958). The calibration curve (s. Fig. 24) was prepared with stercobilin dissolved in saline and then added to dog plasma.

7. a) 6 mg of stercobilin dissolved in 15 ml saline was introduced into an isolated small intestine loop of 15—20 cm long. 15 minutes before injecting, the isolated intestinal segment was irrigated with saline. After 30 minutes the content of the intestine was sucked and examined for urobilinoid.

b) Experimental conditions were also altered by injecting relatively smaller amounts of urobilinoids dissolved in saline 0,2 mg of stercobilin, resp. 1,0 mg and 0,6 mg of i-urobilin were introduced into the isolated small intestinal loop of dogs. After 30—35 minutes the intestinal contents were withdrawn and reinjected into another small intestinal segment of the same dog for another half an hour. 15 minutes before injecting, resp. reinjecting, the isolated intestinal segment was irrigated by saline. The 3 samples (those before and after injecting and after reinjecting) from the 3 experiments were subjected to paper chromatography.

8. At the beginning of the experiment blood was withdrawn from the mesenteric vein and to inhibit coagulation heparin was administered intravenously.

9. The urine was withdrawn directly before and after the experiment with a catheter. In both instances it was attempted to empty the bladder completely.

Results

A) The properties of the stercobilin solution recovered from the intestine

By administrating relatively large amount (6 mg, see methods 7/a) of stercobilin concentration of its solution recovered from the intestine decreased to 63 per cent of the original one. The yielded stercobilin amounted to 3,6 mg. When the native solution recovered from the intestine was submitted to chromatography and subsequently developed with Schlesinger reagent, on the places where it had been applied a green fluorescence characterising urobilinoids could be detected. The spot showing a green fluorescence at the site of application could be stained in the manner characteristic for proteins with acid fuchsin according to NOVOTNY (1952) and with the iron colloid filter paper staining procedure characterising mucopolysaccharides (KAHÁN, Á, 1959). On the chromatogram at the solvent front a red spot can be seen, which, however, does not show an urobilinoid zinc fluorescence if it is sprayed with alcoholic zinc acetate. If the paper strip is heated with hydrochloric acid alcohol according to 5) and then sprayed with Schlesinger reagent the spot located at the front shows a green fluorescence too (Fig. 25 a).

If the recovered intestinal juice was extracted with butanol ammonium sulphate according to HEIKEL et al. (1957), neither a green urobilinoid-zinc fluorescence nor a protein, nor mucopolysaccharide staining were visible on the chromatogram on the site of the application. On the other hand, the chromatogram shows a red spot at the solvent front which, however, exhibits with Schlesinger's reagent a weak green fluores-

cence. Presumably this is a mixture of the already mentioned red urobilinoid derivative and free urobilinoid observed on the chromatogram of the native intestinal juice. The fluorescence of this spot could be enhanced by heating with hydrochloric acid alcohol (Fig. 25 b.).

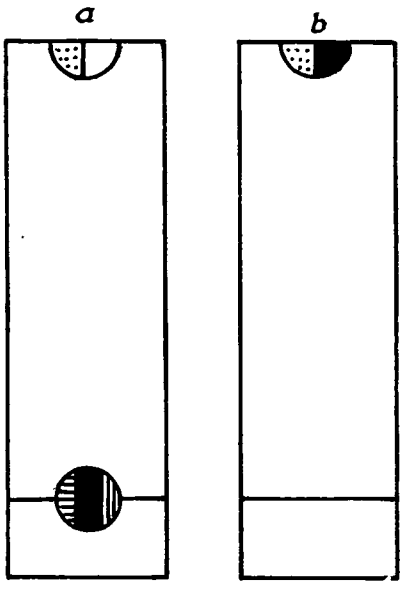


Fig. 25.

The chromatographic fractions of the intestinal juice: a) native intestinal juice; b) chromatogram of urobilinoid solutions obtained by butanol-$(NH_4)_2SO_4$ extraction of the intestinal juice. Fluorescence with Schlesinger's reagent under ultraviolet light: ▬
Colouration with protein staining procedure: ≡
Colouration with mucoprotein staining: ||||
Fluorescence under ultraviolet light after HCl alcohol hydrolysis and spraying with Schlesinger reagent: ⁞⁞⁞

On submitting the stercobilin solution recovered from the intestine to paper electrophoresis two fractions showing a fast mobility rate could be found on the paper strips, that proceeding in front exhibited an intesive red colour and the one with a smaller mobility a weaker one. After spraying with Schlesinger's reagent a weak green fluorescence was found on the site of the simultaneously running stercobilin reference as well as an intensive green fluorescence on the site of the red strips after heating with hydrochloric acid alcohol. With acid fuchsin staining (of proteins) was obtained in the vicinity of the site of application where, however, urobilinoid could not be demonstrated (the urobilinoid split off the proteins and appeared as „free urobilinoid") (Fig. 26.).

If smaller amounts of urobilinoids were introduced into the isolated small intestinal loop (see methods 7/b) only — 10, 5, 25 percent of the introduced urobilinoids could be withdrawn. These relatively small amounts of urobilinoids could be found in a

conjugated form which proved to be immobile in the chromatographic solvent system described above. Reinjecting them into the second isolated intestinal tract, on withdrawal their urobilinoids proved to be unchanged quantitatively and in their chromatographic behaviour, remaining at start-position. However by extracting the withdrawn intestinal contents by $(NH_4)_2SO_4$ — butanol, uncoupled urobilinoids were obtained (Fig. 27.).

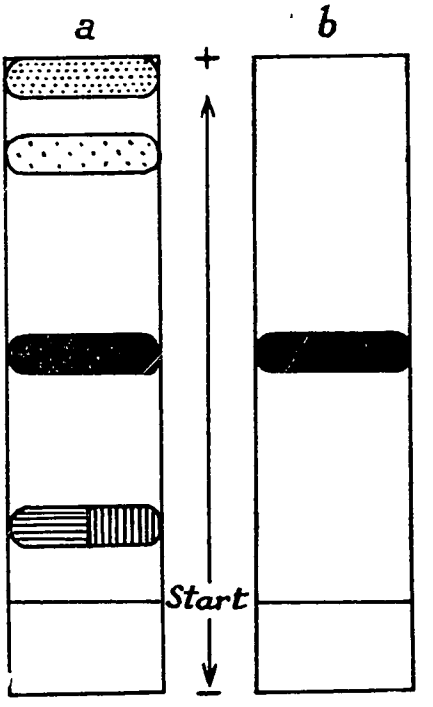


Fig. 26.
Paper electrophoretic fractions of the native intestinal juice (a). For comparison: christalline stercobilin (b).
Fluorescence with Schlesinger's reagent in ultraviolet light:
Colouration with protein staining procedure:
Colouration with mucoprotein staining:
Fluorescence under ultraviolet light after hydrolysis with HCl alcohol and spraying with Schlesinger reagent:

B) Examination of the blood plasma

Fig. 28 shows the amount of stercobilin which gained access into the blood.* It also depicts the change in the plasma stercobilin concentration as a function of time. The curve exhibits the amount of the absorbed stercobilin. The plasma fluor-

* In cases of applying small amounts of urobilinoids (see methods 7/b), they were not demonstrable in blood plasma.

escence values measured before stercobilin administration were subtracted from the fluorescent values measured in the course of the experiment. It may be seen that in the first part of the experiment the stercobilin concentration of the blood plasma increases and reaches its maximum at the 15th minute whilst in the 27th as well as 30th minute it only amounts to 0,2 μg. (Further examinations of the urobilinoid which gained access into the plasma are discussed in chapter 7.)

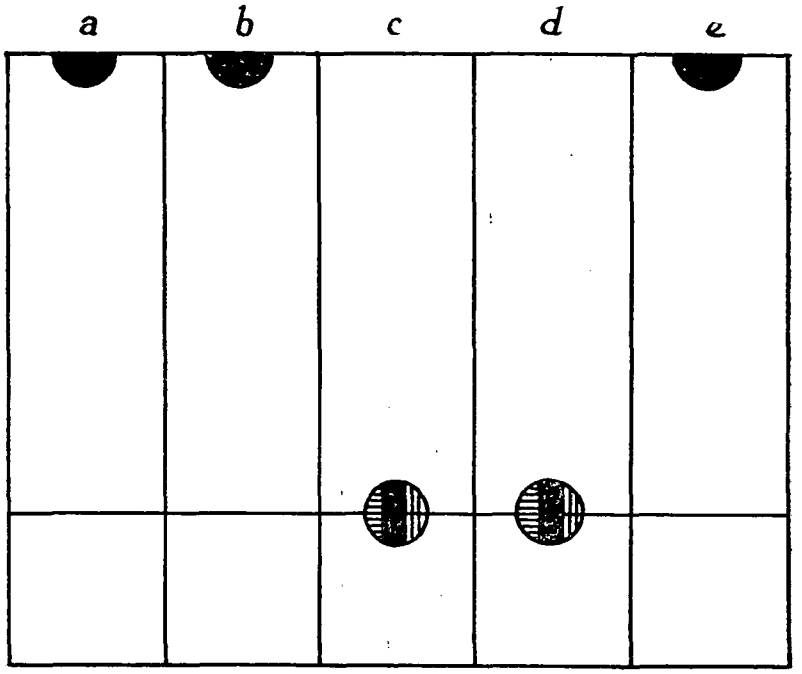


Fig. 27.
The chromatographic fractions of the intestinal juice from
a: First intestinal loop after extraction with $(NH_4)_2SO_4$ — butanol
b: Second intestinal loop after extraction with $(NH_4)_2SO_4$ — butanol
c: First intestinal loop, native form
d: Second intestinal loop, native form
e: for comparison: crystalline stercobilin
Fluorescence with Schlesinger's reagent in ultraviolet light: ■
Colouration with protein staining procedure: ≡
Colouration with mucoprotein staining: ||||

C) Examination of the urine

The urobilinoid content of the urine was tested with Ehrlich's reagent before starting the experiment. Even its concentrated ammonium butanol extract showed with Schlesinger reagent only a weak green fluorescence at the solvent front. With paper electrophoresis urobilinoid could altogether not be detected. (The originally present urobilinoid which could still be demonstrated by chromatography decomposed by the action of the electric current.)

The urine obtained after the experiment showed with Ehrlich's reagent the red colour reaction characterising urobilinogen. On examining it by means of paper chromatography a strong green fluorescence was seen at the solvent front. With paper electrophoresis the simultaneously run crystalline i-urobilin and i-urobilinogen gave

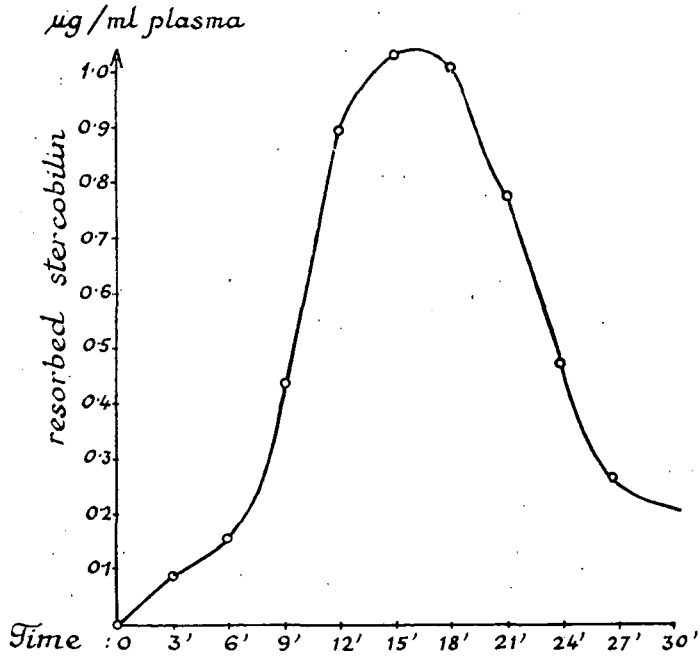


Fig. 28.

Absorption of stercobilin introduced into the intestine.

On the absciss the time on the ordinate the stercobilin quantity measured in the plasma is plotted. Excitation light emission maximum at 473 mµ, fluorescence light measured with filter having an absorption maximum of 553 mµ. Fluorescence light measured mith a galvanometer having a sensitiveness of 5×10^{-9} A/ scale range.

with Schlesinger's reagent or Ehrlich's reagent respectively a positive reaction on the corresponding site. Thus, at least a part of the stercobilin introduced into the intestine is excreted with the urine in the form of its chromogen. (In the solution recovered from the intestine as well as in the plasma urobilinoid chromogen could not be detected with Ehrlich's reagent.)

Conclusions

Before the evaluation of the results I should like to remark that in the above experiment urobilinoid pigment (stercobilin) was introduced into the small intestine, whereas under physiologic conditions the chromogens of the urobolinoids are absorbed from the intestine. As, however, very similar compounds are involved from the above

experimental results conclusions may be drawn concerning the rules relating to the resorption of the chromogens.

As can be seen on the basis of the above mentioned experiments the stercobilin introduced into the small intestine of the dog is reabsorbed. The resorption is, however, stopped not owing to the appreciable decrease of the stercobilin concentration, but as a concequence of some other factors. According to the results of these investigations stercobilin is conjugated to the mucoproteins of the intestine and presumably this mucoprotein bound stercobilin — as a larger molecule — cannot be absorbed through the wall of the intestine.

ROYER (1932) suggests that the urobilinoids are mainly absorbed by the colon and only to a lesser extent by the sigma. Our investigations indicate that the resorption may already take place in an isolated saline washed segment of the small intestine, as in the course of the washing part of the mucoprotein is removed and thus a part of the urobilinoid cannot be bound. Under physiological conditions, however, just owing to the linkage of the mucoproteins of the small intestine the resorption cannot occur in the small intestine, but only in deeper lying sections and it depends on the nature of the protein linkage which part of the intestine the urobilinoids reach. According to WATSON (1959) d-urobilinogen forms in the coecum and i-urobilinogen in the sigma, whilst the reduction to stercobilinogen takes place in a still deeper section.

The fact that the stercobilin introduced into the intestine is excreted with the urine as chromogen corresponds entirely to the fact known since a long time (WATSON, 1936) that the urobilinoid which penetrates into the organism is reduced to its chromogens. Further investigations are needed to establish in which part of the organism this reduction takes place. WATSON (1948—49 b.) suggests that possibly the reduction may take place in the liver as well as in the kidney. Watson attributes to the proteins a certain role at the reduction. The reduction of intravenously administered stercobilin to stercobilinogen is protracted in persons in whom the albumin/globulin quotient is pathologic.

The red fraction of the intestinal juice content — separating in the course of electrophoresis with a rapid mobility — corresponds to urobilinoid bound to some acid component. The colour of the fraction, its rapid mobility and that it only forms a zinc complex after acid hydrolysis (FISCHER, REINECKE, 1939) is in accordance with this assumption.

SUMMARY

Our knowledge about urobilinoids reaches as far back as 100 years. The fluorescence of the zinc complexes of the urobilinoids which is also nowadays often used, was first observed by JAFFÉ and the colour reaction of the urobilinoid chromogens with p-dimethylaminobenzaldehyd by EHRLICH. In spite of this, some questions concerning the structure of the urobilinoids are still under discussion.

Their investigation is rendered difficult by the similarity of their physical and chemical properties and by their easy degradation. One of their physico-chemical properties which is slightly different is that their behaviour in the electric field varies which may be brought into relation with the imaginations concerning the structure of the urobilinoids. The analytical procedure elaborated in this work for the isolated determination of the urinary urobilinoids is based on the difference between the chemical properties of the urobilinoids.

In the organism, however, one cannot only find various urobilinoids, but they also occur in different linkages. Besides free urobilinoid, urobilinoids bound to proteins, mucoproteins, glucosamine and glucuronic acid can be demonstrated in the urine. In addition by the action of the mucoproteins and glucuronidase of the urine an urobilinoid fraction conjugated to neutral oligosaccharides forms too. In the serum the urobilinoids are mainly bound to albumin, but they may also be conjugated to heparin. The urobilinoids in the intestine may also be bound to intestinal mucoproteins.

The methods of these investigations and biochemical knowledge gained through them may furnish a basis for research work on the physiology and pathophysiology of urobilinoids. Though the results belonging to this topic are not described here in extenso clinical observations of 85 cases and determinations of 221 urine samples seem to prove the intestinal origin uf urobilinoids.

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